Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 94 MARCH, 1957 No. 3

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## Suppression of Biosynthesis of Adrenal Cortical Steroids in Man By Amphenone. (22964)

RALPH E. PETERSON, ROY HERTZ, AND HERBERT A. LUBS

*Nat. Inst. of Arthritis and Metabolic Diseases, and Nat. Cancer Inst., N.I.H., U. S. Dept. of Health, Bethesda, Md.*

Amphenone (1,2-bis (*p*-aminophenyl) 2-methyl propanone-1-dihydrochloride), one of a series of substituted desoxybenzoins, was synthesized by Allen and Corwin(1), and observed by Hertz *et al.*(2) to produce a marked enlargement of both adrenals of the rat, associated with an increased deposition of lipid material. In the hypophysectomized animal, amphenone does not produce adrenal enlargement. This suggests that amphenone may act directly on the adrenal to interfere with steroidogenesis, and that the induced adrenal enlargement is secondary to ACTH stimulation. In the hypophysectomized dog(3), amphenone depressed the secretion of 17,21-dihydroxy-20-ketosteroids as measured in adrenal vein blood. This effect was also noted during ACTH stimulation of the adrenal. Hertz *et al.*(4) observed an irregular decrease in urinary and plasma 17,21-dihydroxy-20-ketosteroids, and little or no decrease in excretion of urinary 17-ketosteroids in patients

with breast carcinoma and adrenal hyperplasia. Similar observations were also noted by Thorn *et al.*(5) and Hertz *et al.*(6) in a patient with adrenal carcinoma. Gallagher *et al.*(7) reported decreased urinary excretion of dehydroisoandrosterone, androsterone, etiocholanelone, and the 11-oxygenated analogs of both androsterone and etiocholanelone in a patient with metastatic adrenal carcinoma treated with amphenone.

Because of the failure to obtain lowered urinary 17-ketosteroid values after oral amphenone therapy (except Gallagher *et al.*(7)), and the paradoxical findings in some cases of lowered urinary corticoid excretion without significantly lowered plasma hydrocortisone level, there has been some question as to the possibility that amphenone may have interfered, either with steroid determinations or with rate of hepatic or renal clearance of the steroids. It therefore seemed of interest to further evaluate the effects of amphenone on adrenal function, utilizing more specific methods now available. These methods have also made it possible to obtain data of a quantitative nature relating to the effect of amphenone on adrenal steroid synthesis.

*Methods.* Plasma hydrocortisone and urinary corticosteroids were determined by a modification(8,9) of the Silber-Porter procedure(10). Urinary 17-ketosteroids were determined by a modified Zimmermann procedure(11) that does not require a color correction. Plasma corticosterone was determined by isotope dilution method(12). The turnover rate of hydrocortisone was determined by previously described procedure(13), and the turnover rate of corticosterone by a similar method utilizing the same general principles.

*Results. Effect of amphenone on plasma levels of hydrocortisone and corticosterone.* Oral or intravenously administered amphenone produces a decrease in concentration of both plasma hydrocortisone and corticosterone (Table I). This effect was more pronounced in patients with adrenal carcinoma unresponsive to ACTH but was also observed in normal subjects. In the normal subject, amphenone lowered the plasma steroid levels, even though intravenous ACTH was being administered at 4 units/hour. It is to be noted that the plasma hydrocortisone concentration, as determined colorimetrically with the phenylhydrazine reagent without prior chromatography agreed well with the more specific isotope dilution assay.

TABLE I. Plasma Levels of Hydrocortisone (F) and Corticosterone (B) before and after Amphenone Therapy.

| | | μg % | | |
|---|---|---|---|---|
| | | Phenyl-hydrazine | Isotope dilution | |
| | | F | F | B |
| | *Adrenal carcinoma:* | | | |
| N.G. | Control | 70 | 69 | 1.7 |
| | Amphenone p.o. 6 g q d × 25 | 39 | 38 | .2 |
| V.G. | Control | 156 | — | 2.0 |
| | Amphenone i.v. 1 g q hr × 8 | 46 | 38 | .5 |
| | Post-amphenone | 260 | 240 | 5.6 |
| G.T. | Control | 67 | 60 | 1.7 |
| | Amphenone i.v. 1 g q hr × 8 | 17 | 15 | .5 |
| | *Normal:* | | | |
| H.G. | Control | 19 | 20 | 1.1 |
| | Amphenone p.o. 5 g q d × 5 | 9 | 9 | .4 |
| O.W. | Control (4 units ACTH/hr i.v.) | 69 | 63 | 7.6 |
| | Amphenone i.v. 1.5 g q hr × 4 | 41 | 40 | 2.2 |

*Effect of amphenone on urinary steroid metabolites of hydrocortisone.* Fig. 1 shows the effect of amphenone on urinary steroids in a patient with adrenal carcinoma. One week after oral administration of 6 g/day of amphenone, urinary corticoids fell to within the high normal range. This was associated with a fall in plasma levels of hydrocortisone, but to concentrations that were still 2 to 3 times normal. Urinary steroids did not show a further decline after 12 days of treatment. There was no significant fall in total urinary 17-ketosteroids; however, paper chromatographic studies showed that there was a marked decline in level of the 11-oxygenated 17-ketosteroids. These steroids presumably arise as metabolites of hydrocortisone.

*Effect of amphenone on rate of metabolism of steroids.* The rate of disappearance of

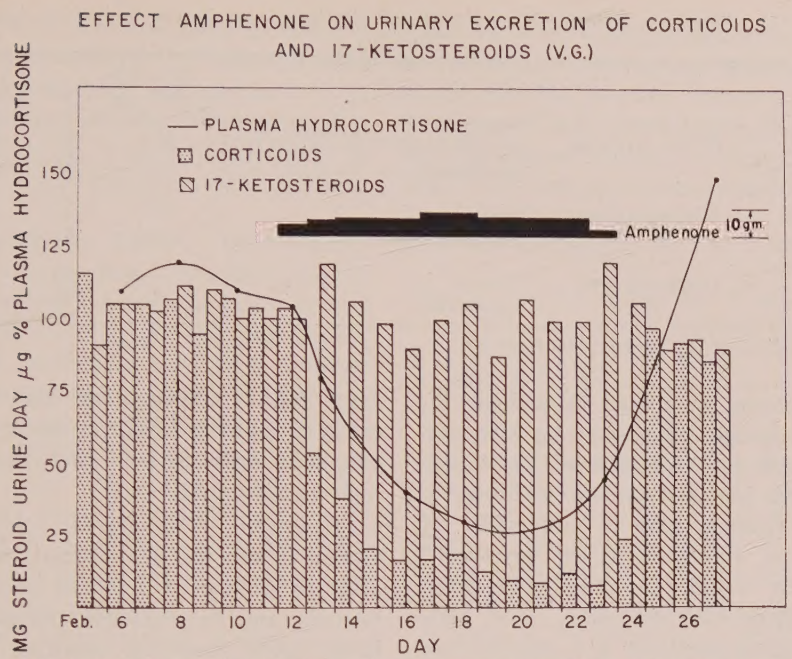


FIG. 1. Effect of orally administered amphenone on concentrations of urinary corticoids and 17-ketosteroids, and plasma hydrocortisone.

intravenously infused hydrocortisone or corticosterone was not altered after administration of amphenone to normal subject (Table II). Also, the rate of disappearance of tetrahydrohydrocortisone, which presumably is a measure of ability of liver to conjugate steroids, was not delayed.

*Effect of amphenone on rate of synthesis of hydrocortisone and corticosterone.* In patient G.T., with metastatic adrenal carcinoma, the miscible pool and rate of synthesis of hydrocortisone was increased approximately 8-fold over the normal(13) (Table III). The corticosterone pool and turnover rate was increased approximately 2.5 times normal (unpublished observations). Following amphenone therapy, the pools and turnover rates of both hydrocortisone and corticosterone decreased to within normal range. In the normal subject under the influence of maximal exogenous ACTH stimulation, intravenously administered amphenone resulted in a decrease in both pool size and rate of synthesis of hydrocortisone; however, the depression was not as great as in the patient with adrenal carcinoma.

*Evaluation of effect of amphenone and its metabolites on the method of assay.* The fol-

TABLE II. Biological Half-Times of Steroids before and after Amphenone Therapy.

| | | $t_{1/2}$ minutes | | |
|---|---|---|---|---|
| | | Hydrocortisone | Corticosterone | Tetrahydrohydrocortisone |
| V.S. | Control | 120 | | 35 |
| | Amphenone i.v. 1 g q hr × 4 | 128 | | 48 |
| H.G. | Control | 110 | | 43 |
| | Amphenone p.o. 5 g q d × 6 | 110 | | 33 |
| O.W. | Control | — | | 26 |
| | Amphenone i.v. 2.5 g q hr × 3 | — | | 27 |
| T.R. | Control | | 90 | |
| | Amphenone p.o. 4 g q d × 4 | | 78 | |

TABLE III. Turnover Rate Hydrocortisone and Corticosterone before and after Amphenone Therapy.

| | | | Miscible pool, mg | K* | mg/hr |
|---|---|---|---|---|---|
| O.W. Normal | Hydrocortisone | Control (4 units ACTH/hr i.v.) | 15.3 | .53 | 8.1 |
| | | Amphenone, i.v., 1.5 g q hr × 6 | 9.2 | .44 | 4.1 |
| G.T. Adrenal carcinoma | Hydrocortisone | Control | 12.2 | .72 | 8.8 |
| | | Amphenone, i.v., 1 g q hr × 8 | 2.0 | .51 | 1.0 |
| | Corticosterone | Control | .52 | .55 | .29 |
| | | Amphenone | .22 | .59 | .13 |

* Fraction of the pool replaced per hr.

lowing procedures were used to evaluate the possible interference of amphenone with steroid determinations: Addition of hydrocortisone to plasma from patient who had received amphenone (plasma level of amphenone 20 μg %) and in whom the plasma hydrocortisone was zero[1] resulted in quantitative recovery of the added hydrocortisone. Also, addition of tetrahydrocortisone and androsterone to urine of patient maintained on 6 g of amphenone/day showed no interference with recovery of the steroids as determined by phenylhydrazine or Zimmermann procedures.

*Interference of amphenone and its metabolites with color reaction for steroids.* Two g amphenone were given orally to a subject in whom the plasma hydrocortisone concentration was zero. Plasma samples were taken, 1, 2, and 4 hours after administration of the amphenone and assayed for hydrocortisone by the phenylhydrazine procedure. No hydrocortisone-like material was measured. Also, the close agreement of plasma hydrocortisone levels as determined by the phenylhydrazine procedure and isotope dilution method (Table I) indicated that amphenone or its metabolites did not contribute any significant amount of color with the phenylhydrazine reagent. Six g of amphenone/day were given to patient who had urinary corticoid level of <0.5 mg/day and urinary 17-ketosteroid level of 2 mg/day. The amphenone medication did not cause an increase in chromogenic material as measured with either phenylhydrazine or Zimmermann reagents.

*Discussion.* The lowered plasma steroid levels, failure to demonstrate any impairment in ability of liver to metabolize the steroids, and the decreased urinary corticosteroid levels obtained following administration of amphenone all indicated that amphenone affected rate of synthesis of hydrocortisone and corticosterone by the adrenal cortex. The results of the isotope dilution method of assay, because of its specificity, would tend to rule out any possibility of an artifactual lowering or elevation of these levels through interference of amphenone with the assay procedure. The isotope dilution assay has served to confirm the general reliability of the modified Silber-Porter phenylhydrazine colorimetric assay for determination of hydrocortisone in plasma of patients receiving amphenone. It is also of interest to note that in the baseline control plasmas of patients with adrenal carcinoma, the colorimetric method gives essentially the same plasma hydrocortisone concentrations as the isotope dilution method. This would indicate that essentially all free 17,21-dihydroxy-20-ketosteroid in the plasma was hydrocortisone. In general, the plasma hydrocortisone and corticosterone levels decreased to the same degree in all subjects given amphenone.

The results of the studies on phenylhydrazine colorimetric method for plasma and urine and the Zimmermann color method on urine containing amphenone and its metabolites afford no evidence to indicate that amphenone

[1] Plasma hydrocortisone levels of zero and urine corticosteroid levels of <1.0 mg/day were produced by oral administration of 2 mg of $\Delta^{1}$,9α-fluorohydrocortisone/day in divided doses for one or more days. Urinary 17-ketosteroid values of <3 mg/day were produced after 3 or more days of adrenal suppression with $\Delta^{1}$,9α-fluorohydrocortisone.

therapy interfered with the assay in either a positive or negative manner. Thus, decreased levels of corticoids would not appear to be of a spurious nature, and the failure to find a marked decrease in urinary ketosteroids in these patients would appear to be a valid observation.

After amphenone therapy, the miscible pool and rate of synthesis of hydrocortisone and corticosterone were decreased. In the normal subject receiving ACTH, the depressant effect of amphenone was not as marked as in the patient with adrenal carcinoma, whose adrenal tumor was relatively unresponsive to ACTH. Also, decreased rate of synthesis of hydrocortisone observed in the normal subject given ACTH, indicates that amphenone may exert its suppressive effect directly on the adrenal, rather than through suppression of the pituitary.

It thus seems apparent that amphenone affects synthesis of corticosteroids by the adrenal rather than affecting their rate of metabolism. The clinical observations in patients with adrenal carcinoma given amphenone (*viz.* fall in the elevated blood pressure and hyperglycemia, and a decrease in glycosuria and insulin requirement(4,5)) substantiate the laboratory data on adrenal cortical steroid synthesis.

*Summary.* Administration of amphenone to patients with adrenal carcinoma and to normal subjects caused a fall in concentration of plasma hydrocortisone and corticosterone, and urinary 17,21-dihydroxy-20-ketosteroids. No striking decrease in urinary 17-ketosteroids was noted after amphenone therapy. The rate of metabolism of hydrocortisone, corticosterone, and tetrahydrohydrocortisone was not altered by amphenone therapy. Studies on the miscible pool and turnover rate of hydrocortisone and corticosterone demonstrated that amphenone exerted its effect through inhibition of adrenal cortical synthesis, and this was more marked with adrenal tissue relatively unresponsive to ACTH.

We are indebted to Miss Aurora Karrer and Mr. George Nokes for valuable technical assistance.

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Received December 28, 1956. P.S.E.B.M., 1957, v94.

# Method for Determination of Urinary Pregnane-3-Alpha, 17-Alpha 20-Alpha Triol.* (22965)

WALTER HERRMANN AND LILLIAN SILVERMAN
(Introduced by P. K. Bondy)
*Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, Conn.*

Butler and Marrian(1) isolated pregnanetriol from urine of 2 women with adrenogenital syndrome. Bongiovanni(2) showed that increase in pregnanetriol was the most striking abnormality in urine steroid pattern of children with congenital adrenal hyperplasia. The method for determination of urinary pregnanetriol as proposed by Bongiovanni(3) included enzyme hydrolysis, chromatography on alumina, with final determination of pregnanetriol as sulfuric chromogen. This method has given us rather inconsistent results. Recovery experiments with authentic pregnanetriol showed that the steroid was partially and inconsistently destroyed by adsorption on alumina. Furthermore, when the method was applied to urinary extracts, extraneous material present in the eluate gave rise to color in sulfuric acid and interfered with quantitative determination of the sulfuric acid chromogen.

In the modification described here, urine extract is chromatographed on florisil. The eluate containing pregnanetriol is oxidized with bismuthate according to the method of Norymbersky(4,5) and the pregnanetriol measured as the corresponding 17-ketosteroid.

*Materials.* All reagents were analytical grade and all solvents redistilled. Beef liver beta-glucuronidase was used for hydrolysis. The preparation of florisil was slightly modified from that of Eik-Nes, Nelson and Samuels(6), in that florisil was cooled to 120°C and kept at this temperature. *Methods. Hydrolysis and Extraction.* Aliquots of either 50 or 100 ml were taken from freshly collected 24-hour urine samples. The pH was adjusted to 4.5 (Hydrion test paper) with glacial acetic acid; acetate buffer (0.5 ml/5 ml of urine) and beta-glucuronidase (300 units/ml) were added and urine incubated at 37°C for 48 hours. The urine was extracted twice with equal volumes of ether and then discarded. The combined ether extracts were washed with 0.1 normal sodium hydroxide until pigment free, and with water until neutral, and then dried under nitrogen. Traces of water were removed with benzene and ethanol. *Chromatography.* A florisil column measuring 1 cm diameter and 8 cm in height and supported by small plug of glass wool, was prepared in benzene. The extract was quantitatively transferred to the column with benzene. Slight heating was occasionally required. Elution was carried out using 75 ml portions of 1, 2, and 4% of ethanol in benzene. The 17-ketosteroids and pregnanediol were eluted in the 2% fraction, and the pregnanetriol is contained in the 4% fraction, while the more polar steroids remain on the florisil column. After chromatography the fraction containing pregnanetriol was dried under nitrogen using temperatures lower than 60°C. The residue was dissolved in 10 ml absolute ethanol from which two 4 ml aliquots were taken, one to be oxidized (A), the other (B) to be dried as unoxidized blank for color reaction. *Oxidation and colorimetry.* The method as described by Norymbersky(4,5) was used to convert pregnanetriol to 17-ketosteroid. Sample A was dried and taken up in 8 ml of 50% acetic acid. One gram sodium bismuthate was added, followed by shaking in the dark for 30 minutes. Ten ml of 6% sodium pyrosulfate ($Na_2S_2O_5$) were added and shaking continued for 15 minutes to reduce the remaining bismuthate. After adding 18 cc of water, the acid solution was transferred to a separatory funnel, extracted twice with ether, which was washed with 5% sodium hydroxide until alkaline, and with water until neutral. Ether was dried over sodium sulfate then transferred to 50 cc tapered centrifuge tube and evaporated. For color development a Holtorff and Koch

* This work was supported by grant from U. S. Public Health Service.

TABLE I. Recovery of Pregnanetriol from Florisil Column. (90 μg pregnanetriol adsorbed on column.)

| Recovered in 4% ethanol/benzene, μg | Recovery, % |
|---|---|
| 79 | 88 |
| 77 | 85 |
| 77 | 85 |
| 78 | 86 |

(7) micromethod was used (1% metadinitrobenzene, 5 N aqueous KOH). The residue was taken up in 0.4 ml of metadinitrobenzene, 0.2 ml of alkali were added and the sample incubated 60 minutes in the dark. This procedure was carried out simultaneously on the unoxidized sample (B). The amount of 17-ketosteroid obtained from pregnanetriol was calculated by subtracting the Zimmermann value of sample B from that of sample A, using dehydroisoandrosterone as standard, and multiplying by the ratio of molecular weights

$$\frac{\text{(Molecular wt pregnanetriol)}}{\text{(Molecular wt etiocholanolone)}}.$$

*Results.* Studies were undertaken to determine possible sources of loss of steroid: A. Recovery following chromatography on florisil column: Adequate separation of pregnanetriol from steroids of similar polarity was determined by absorbing 2 mg each of 11$\beta$ - hydroxyetiocholanolone, pregnane - 3$\alpha$, 17$\alpha$, 20$\alpha$ triol-11, 20-dione (tetrahydro E) and 1 mg of pregnanetriol on the florisil column and eluting these by the method described. All the Zimmermann material was eluted with 2% ethanol-benzene solution. No Porter-Silber chromogen was found in the 4% fraction but was eluted in 8% ethanol-benzene. In another experiment pregnanetriol as determined by its sulfuric acid chromogen, was recovered as indicated in Table I. B. Recovery following oxidation procedure: Varying amounts of crystalline pregnanetriol were converted to etiocholanolone by the oxidation procedure. The pregnanetriol equivalent recovered is shown in Table II. C. Total recoveries from water and from urine are shown in Table III. A series of urines from 12 normal females, 4 normal males and one patient with adrenogenital syndrome was also examined (Table IV).

TABLE II. Recovery of Pregnanetriol following Oxidation.

| Pregnanetriol, μg | Pregnanetriol calculated from recovered etiocholanolone, μg | Recovery, % |
|---|---|---|
| 48 | 44 | 92 |
| 96 | 91 | 95 |
| 28 | 28 | 100 |
| 80 | 64 | 80 |

TABLE III. Total Recoveries of Pregnanetriol (5 Normal Subjects).

| Material, ml | Endogenous pregnanetriol | Added pregnanetriol, μg | Recovery of added pregnanetriol, % |
|---|---|---|---|
| $H_2O$, 50 | | 90 | 82 |
| Urine, 50 | 70 | 90 | 85 |
| *Idem* | 65 | 90 | 70 |
| " | 83 | 90 | 73 |
| " | 0 | 90 | 83 |
| " | 15 | 90 | 83 |

*Conclusion.* A method for determination of pregnanetriol is presented. The method seems to be reproducible with adequate recoveries and specificity. Recoveries average 78.5%. Specificity is assured by chromatographic purification, oxidation, and determination of pregnanetriol as ketogenic steroid. The spectrum of material from the florisil column in sulfuric acid was authentic with the one obtained from pure pregnanetriol (Fig. 1). In the oxidized sample the Zimmermann color obtained was purple with distinct peak at 520 mμ (Fig. 2). The un-

TABLE IV. Excretion of Pregnanetriol from Normal Patients 21 to 31 Years Old.

| | Pregnanetriol per 24 hr, mg | Pregnanetriol per 24 hr, mg |
|---|---|---|
| Normal ♀ | 1.5 | .54 |
| | 1.3 | .39 |
| | 1.3 | .80 |
| | .0 | 1.67 |
| | 1.1 | 1.01 |
| | 1.36 | 1.6 |
| Normal ♂ | 2.4 | |
| | 1.3 | |
| | 1.4 | |
| | 2.3 | |
| Adrenogenital syndrome (♀) | 16. | |

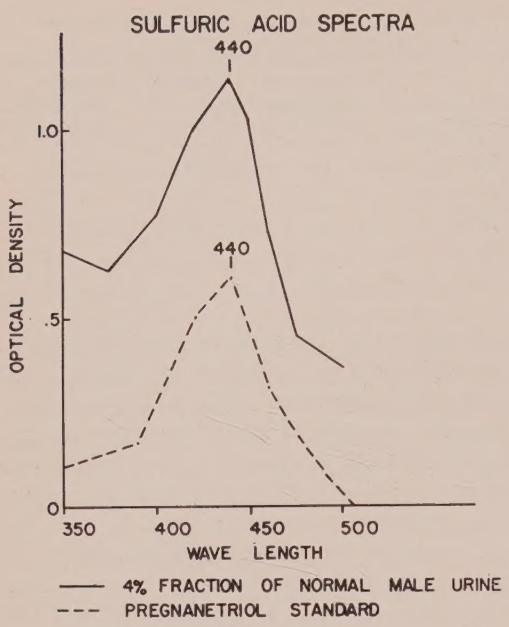


FIG. 1.

oxidized sample showed brown color with the Zimmermann reagent and gave a linear absorption curve.

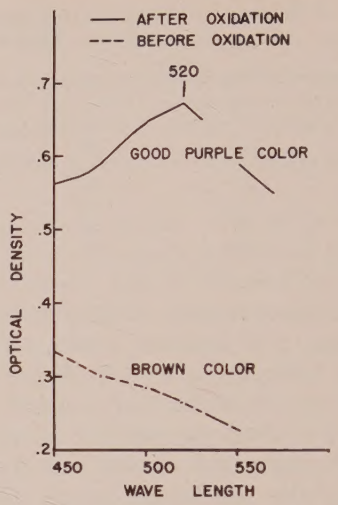


FIG. 2. Spectrum of oxidized and unoxidized 4% fraction following Zimmermann reaction (normal male urine).

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Received October 19, 1956. P.S.E.B.M., 1957, v94.

## Blood 5-Hydroxytryptamine (Serotonin) Levels after Reserpine and Electroshock Therapy.* (22966)

JACK PETER GREEN, MATTI K. PAASONEN,† NICHOLAS J. GIARMAN
(Introduced by D. D. Bonnycastle)
*Department of Pharmacology, Yale University School of Medicine, New Haven, Conn.*

Recent evidence appears to lend credence to the postulate of Gaddum(1) and Woolley and Shaw(2) that 5-hydroxytryptamine (5-HT, serotonin) is involved in the function of the nervous system. Shore, Silver, and Brodie have shown that animals treated with the tranquilizing drug, reserpine, excrete large quantities of 5-HT metabolite, 5-hydroxyindoleacetic acid(3), and that brain and other body stores of such animals become depleted of their 5-HT(4,5). They have demonstrated further that this 5-HT-releasing activity is limited only to those Rauwolfia alkaloids with tranquilizing action(6).

It seemed of interest to determine blood levels of 5-HT in human subjects after treatment with reserpine; at the same time we

* Aided by grant from Nat. Institutes of Health.

† Riker and Fulbright Fellow in Pharmacology from University of Helsinki.

studied the effect of electro-convulsive therapy on blood levels of 5-HT. Since permeability of the so-called blood-brain barrier is increased (at least in certain respects) after electroshock(7), it was conceivable that a change in concentration of 5-HT in the brain might be reflected by concentrations in blood. Blood levels of 5-HT were chosen rather than urinary levels of 5-hydroxyindoleacetic acid because the latter do not always reflect alterations in blood levels(8).

*Methods.* All patients were white males in a neuropsychiatric ward. Blood, usually 3 cc, was obtained from the antecubital vein, mixed with sodium citrate and extracted for one hour with 20 volumes of 99% acetone, according to the method of Amin *et al.*(9). The mixture was filtered, the acetone extract brought to dryness *in vacuo* and kept overnight below 0°C if not analyzed immediately. 5-HT was determined on the isolated heart of *Venus mercenaria*‡(10). The nutrient fluid for the heart contained (g/l): NaCl, 23.0; $Na_2SO_4$, 4.0; KCl, 0.65; $MgCl_2 \cdot 6H_2O$, 5.0; and $NaHCO_3$, 0.2(11). Benzoquinonium chloride ("Mytolon," Sterling-Winthrop) was always present in concentration of 6 mg/l to block the effect of any acetylcholine present in the extract. In our hands this method of extraction and assay has yielded reliable and reproducible estimates of serotonin levels in a number of biological tissues. Recoveries of added quantities of serotonin have ranged from 75% to 102%. However, insofar as whole blood is concerned, the amounts of 5-HT obtained by our extraction procedure must now be considered of relative and not of absolute significance, since Hardesty and Stacey have shown that erythrocytes interfere with the total extraction of serotonin from blood(12). Reliability of the bioassay is demonstrated in part by the fact that the extracts made in this work produced dose-response curves on the heart of *Venus mercenaria* identical to those produced by known quantities of serotonin. This bioassay preparation is highly sensitive to and specific for the stimulatory action of serotonin, since other cardio-excitatory amines like epinephrine, nor-epinephrine and histamine fail to stimulate it. Control studies with chlorpromazine (in concentrations up to 1 μg/ml) and reserpine (up to 10 μg/ml) indicated that these substances are without effect upon contractility and rate of the Venus heart and without influence upon its response to serotonin. Of 10 patients given reserpine, 4 were taking the drug when the experiments began. They were taken off reserpine for 3 weeks during which time all other medications were continued. To 6 other patients, reserpine was added to their previous medications. Three collections of blood were made on patients receiving electro-shock therapy: one immediately before and one immediately after administration of pentothal and succinylcholine; the third immediately after electro-shock. Since platelets carry blood 5-HT(12), platelet counts were done, but these did not vary significantly among patients or over the course of the experiment.

*Results.* The first 6 patients listed in Table I were not on reserpine at beginning of experiment. Their blood levels of 5-HT varied from 3.5 to 14 μg/100 ml. After 3 weeks on reserpine, 5-HT was minimal or not detectable in their blood.

The 5-HT levels in these patients before reserpine administration were within the wide range observed in this and other laboratories (13) in normal males and non-psychiatric patients with the exception of the value of 14 μg/100 ml observed in number 4, a patient with peripheral vascular disease. Three other patients with schizophrenia also fell within the range 2.3-8.5 μg/100 ml.

The last 4 patients listed in Table I were taking reserpine as well as other drugs when the experiments were begun. 5-HT was not detectable in their blood at this time. Three weeks after reserpine was withdrawn 5-HT levels had reached 1.7-2.5 μg/100 ml, values still appreciably below the normal range.

The concentration of 5-HT in the blood of 3 patients under electro-shock therapy was determined. In none did the initial treatment

‡ We are indebted to V. L. Loosanoff of the U. S. Fish and Wildlife Service at Milford, Conn. for his cooperation in supplying *Venus mercenaria* for this study.

TABLE I.

| Age | Diagnosis | Initial therapy (daily) | 5-HT levels, μg/100 ml | Therapy for 3-wk interval (daily) | 5-HT levels, μg/100 ml |
|---|---|---|---|---|---|
| | | mg | | mg | |
| 60 | Schizophrenia, hebephrenic | 0 | 2.8 | 3.0 reserpine | <.11 |
| 34 | Schizophrenia, unclassified | 300 thorazine | 5.0 | 300 thorazine 1.5 reserpine | .11 |
| 29 | Schizophrenia, hebephrenic | 450 " | 4.0 | 450 thorazine 1.5 reserpine | <.11 |
| 40 | Schizophrenia, paranoid | 300 " | 14.0 | 300 thorazine 3.0 reserpine | " |
| 67 | Meningoencephalitic syphilis | 0 | 8.3 | 3.0 " | .14 |
| 70 | Paresis | 300 dilantin | 3.5 | 300 dilantin 3.0 reserpine | <.17 |
| 35 | Schizophrenia, unclassified | 150 thorazine .75 reserpine | < .25 | 150 thorazine | 2.5 |
| 41 | Schizo-affective disorder with depression | 75 thorazine 1.5 reserpine | <1.0 | 75 " | 2.4 |
| 32 | Schizophrenia, hebephrenic | 450 thorazine 2.25 reserpine | < .4 | 450 " | 1.7 |
| 59 | Paresis | 300 dilantin 6.0 reserpine | < .45 | 300 dilantin | 2.2 |

affect the level of 5-HT in the blood. One 40-year-old patient with a reactive depression was followed for a 2-week period during which electro-shock was administered 6 times; there was no significant change in blood 5-HT levels over this period. Another patient on reserpine therapy (T.G. in Table I) was followed for 3 weeks, (9 electro-shock treatments) with no detectable change in concentration of 5-HT in the blood.

*Summary.* Blood 5-HT fell to non-detectable levels after administration of reserpine to patients during a 3 week period. Three weeks after withdrawal of the drug, 5-HT levels had risen but had not yet reached normal values. Electro-shock therapy did not alter the levels of 5-HT in the blood.

We are grateful to Lane Ameen of Neuropsychiatric staff of West Haven Veterans Administration Hospital for access to these patients and to the staff for their willing cooperation.

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Received November 8, 1956. P.S.E.B.M., 1957, v94.

# Effect of Cortisone on Treatment of Tetanus with Antitoxin. (22967)

TE-WEN CHANG AND LOUIS WEINSTEIN

*Haynes Memorial and Evans Memorial of the Mass. Memorial Hospitals, and Department of Medicine, Boston University School of Medicine, Boston, Mass.*

Studies of the effect of various materials derived from the adrenal on the activity of bacterial products have yielded variable results. With diphtheria toxin, increase in susceptibility(1,2), no effect(3), and increased resistance(4,5,6) have been noted when adrenal substances were administered. Favour(8) reported that cortisone increased the susceptibility of guinea pigs to small quantities of diphtheria toxin, but raised the level of resistance to larger doses. Cortisone was noted by Brainerd and co-workers(7) not to interfere with the neutralization of diphtheria toxin by specific antitoxin.

Although studies of cortisone in tetanus intoxication in animals have indicated little or no effect(5,9,10), it has been suggested that the steroid exerts a favorable action in human tetanus(11,12). There are no reports in the literature concerning the activity of cortisone on neutralization of tetanus toxin by antitoxin in controlled experiments. It is the purpose of this paper to present evidence which indicates that cortisone interferes with the therapeutic activity of tetanus antitoxin, and enhances the effect of toxin in unprotected animals.

*Methods.* Highly purified tetanus toxin (0.005 mg contained 1 M.L.D. for mice) prepared by the Biologic Laboratory of Mass. State Dept. of Health, was diluted in heart infusion broth (pH 6.6), and 1 M.L.D. injected intramuscularly over the right hind leg of mice (groups of 9) weighing 20 ± 2 g. Antitoxin, a pepsin-digested immune horse serum, was administered intraperitoneally at varying times in a dose of 0.5 unit in a volume of 0.2 ml; 0.001 unit of this antitoxin neutralized 5-20 mouse M.L.D. of the toxin used in the study. Cortisone acetate (Cortone, Sharp and Dohme) was diluted with normal saline and 2 mg injected subcutaneously 4 hours and again 20 hours after the antitoxin. Control animals (groups of 9) received toxin or steroid alone. Deaths in each group were counted daily for 10 days. The treatment schedule used is described in Table I.

*Results.* The results obtained in these experiments are indicated in Fig. 1. When antitoxin was given 24 hours after injection of toxin, the death rate in 10 days was 11.1%. However, when cortisone was administered 4 hours after antitoxin, the number of fatalities was increased to 55.5%. Treatment with antitoxin 48 hours after tetanus toxin led, as could be predicted, to a higher incidence of deaths (55.5%), but even at this time the steroid neutralized the antitoxic effect to some degree, 77.7% of the treated animals succumbing to the intoxication. When antitoxin was delayed for 72 hours, all of the mice died within 10 days, without relation to the use of steroid. In the control animals receiving toxin alone, the fatality rate after 5 days was smaller (55.5%) than in those given toxin plus cortisone (88.8%). At the end of 10 days, however, the difference, although still present, was smaller. The average survival time with toxin alone was 82.5 hours while with toxin and cortisone, it was 69.5 hours. That the hormone in the quantity used was not itself responsible for death is illustrated by the fact that all of the mice treated with the drug alone remained alive.

*Discussion.* The phenomena which have been observed when cortisone has been ad-

TABLE I. Schedule of Treatment.

| Group No. | Hours 0 | 24 | 28 | 48 | 52 | 72 | 76 |
|---|---|---|---|---|---|---|---|
| 1 | T | A.T. | E | | E | | |
| 2 | T | " | | | | | |
| 3 | T | | | A.T. | E | | E |
| 4 | T | | | " | | | |
| 5 | T | | S | | S | | |
| 6 | T | | E | | E | | |
| 7 | | | E | | E | | |

T, tetanus toxin; A.T., antitoxin; E, cortisone; S, .85% saline.

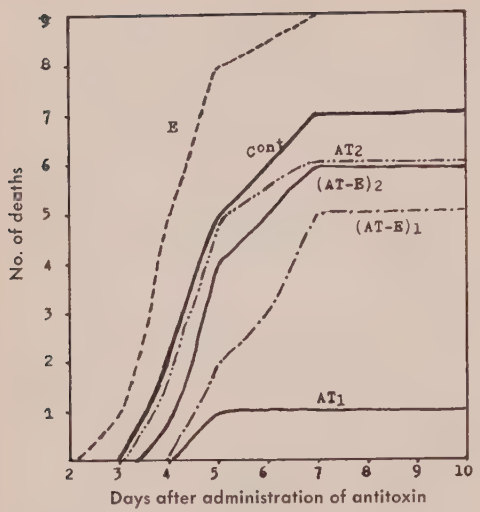


FIG. 1. Effect of cortisone on tetanus toxin and antitoxin. E, cortisone; Cont., no antitoxin or cortisone (toxin alone); $AT_1$, antitoxin 24 hr after toxin; $(AT\text{-}E)_1$, antitoxin and cortisone 24 hr after toxin; $AT_2$, antitoxin 48 hr after toxin; $(AT\text{-}E)_2$, antitoxin and cortisone 48 hr after toxin.

ministered to animals injected with various bacterial toxins appear to be influenced to a great degree by time relationships of administration of drug and exotoxin(10). The relative quantities of toxin and hormone are also of importance in determining the results(4, 5,7,8). The experiments described in this paper were designed to simulate to some degree the situations which might arise in clinical practice. For this reason, antitoxin alone and this agent plus cortisone were given 24, 48 or 72 hours after first exposure to tetanus toxin. The quantity of antibody used was very large (0.5 unit neutralized between 2500 and 10,000 M.L.D. of toxin). Despite the greatly excessive dose of antibody, the depressing effect of steroid on protection was still marked when antitoxin was administered at a time when in animals, not receiving cortisone, a distinct therapeutic effect was produced. With relatively late injection of antitoxin, little or no difference was observed when cortisone was exhibited because the immune material itself was without effect. In addition, as can be seen in the graph, steroid therapy enhanced the activity of the toxin. It is quite possible that a different result might have been obtained had the animals been given antitoxin prophylactically. Brainerd(7), for example, has demonstrated that cortisone does not interfere with the neutralizing effect of diphtheria antitoxin when it is administered 4 days before toxin. This is not the situation which pertains, however, in the management of humans in whom there may be a relatively long period of delay between wounding and prophylaxis, or in whom active tetanus is already present and antitoxin is given therapeutically. The present study suggests that in the treatment of tetanus in which clinical signs have already appeared, or in instances in which a relatively long period of time has elapsed before prophylaxis, the administration of cortisone is not only without benefit but, in fact, sharply decreases the effectiveness of the antitoxin, as well as increases the activity of the toxin which is either fixed or still circulating.

*Conclusions.* 1. The therapeutic effectiveness of very large doses of tetanus antitoxin is markedly reduced by simultaneous administration of cortisone, when both agents are given 24 hours after injection of toxin. 2. Administration of steroid has little or no effect on the course of experimental tetanus if administration of antitoxin is delayed 48 or more hours after exposure to toxin. 3. Cortisone increases the activity of tetanus toxin. 4. Results suggest strongly that the use of cortisone in addition to antitoxin for treatment of human tetanus may produce a deleterious effect.

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Received November 28, 1956. P.S.E.B.M., 1957, v94.

## LSD-Like Effects Elicited by Reserpine in Rabbits Pretreated With Iproniazid.* (22968)

PARKHURST A. SHORE AND BERNARD B. BRODIE

*Laboratory of Chemical Pharmacology, National Heart Institute, Bethesda, Md.*

Previous findings have led us to postulate that the sedative and parasympathomimetic effects of reserpine are mediated through serotonin, and that serotonin may be a chemical transmitter in the parasympathetic division of the central autonomic nervous system (1,2). These concepts are based in part on the findings that both serotonin and reserpine induce sedative effects in mice and potentiate by a central mechanism the action of certain hypnotics, the potentiation by either substance being antagonized by pretreatment with lysergic acid diethylamide (LSD); and that the prolonged effects of reserpine are associated with a persistent impairment of the capacity of the body to maintain serotonin in the bound form which normally protects the amine from the action of the active enzyme, monoamine oxidase. As a consequence, serotonin is liberated and rapidly metabolized, but as the enzyme in brain responsible for formation of serotonin remains unimpaired, the possibility has been considered that reserpine administration results in a persistent flow of serotonin from cells which have lost part of their capacity for storing the indole. The total level of serotonin is low, as would be expected because free serotonin is rapidly metabolized by monoamine oxidase, but the free amine might stimulate the central parasympathetic centers, thus producing the signs typical of reserpine.

The present paper describes studies which demonstrate that when reserpine is administered to rabbits pretreated with the monoamine oxidase inhibitor, iproniazid, the animals, instead of showing sedative and parasympathomimetic responses typical of reserpine, display instead the excitatory and sympathomimetic effects typical of lysergic acid diethylamide (LSD). Furthermore, the concentration of serotonin in brain, which declines rapidly after the administration of reserpine alone, now remains at its normal level.

*Methods.* Male albino rabbits were given various drugs intravenously and were killed by intravenous injection of air one hour after administration of the last drug. Serotonin in brain was assayed by the fluorometric procedure described by Bogdanski *et al.*(3).

*Results.* Five rabbits given reserpine (5 mg/kg) demonstrated typical responses to the drug including deep sedation and parasympathomimetic effects including miosis and relaxation of nictitating membrane. Five animals pretreated with 100 mg/kg of iproniazid and 2 hours later given 5 mg/kg of reserpine showed no parasympathomimetic effects, but rather displayed gross excitation, mydriasis, exophthalmus, piloerection, and other sympathomimetic effects. Animals given iproniazid alone, displayed no obvious signs.

It seemed possible that the sympathomimetic effects observed following the administration of reserpine to iproniazid-treated rabbits were caused by the drug combination rather than by serotonin which had been released by reserpine and protected by iproniazid from the action of monoamine oxidase. This possibility was investigated with another series of 5 rabbits in which the order of drug administration was reversed; that is, the ani-

* Presented in part before the American Soc. for Pharmacol. and Exp. Therap., Iowa City, Sept. 1955.

TABLE I. Effect of Iproniazid on Concentration of Serotonin in Brains of Normal and Reserpine-Treated Animals.

Rabbits were given reserpine (5 mg/kg) intrav. Iproniazid (100 mg/kg) was admin. intrav. 2 hr before or after reserpine. Animals were sacrificed one hr after admin. of the last drug. Each value represents a single animal.

| Drugs admin. | Brain serotonin, μg/g | Effect |
|---|---|---|
| None | .51, .53, .62, .54, .55 | |
| Iproniazid | .60, .67, .63, .62 | None |
| Reserpine | .08, .12, .10, .12, .08 | Sedation |
| Iproniazid followed by reserpine | .40, .44, .72, .56, .55 | Excitement |
| Reserpine followed by iproniazid | .18, .16, .10, .11, .06 | Sedation |

mals were given reserpine followed 2 hours later by iproniazid. All of these animals were sedated and showed parasympathomimetic signs associated with reserpine administration.

In Table I are compared the serotonin levels in brains of normal animals and of animals given the various drugs. The serotonin level in whole brains of animals given reserpine alone declined from about 0.55 to about 0.1 μg/g of tissue. Iproniazid alone had only a slight effect on the concentration of serotonin in the time period of these experiments. Brains of animals given iproniazid followed by reserpine showed about the same concentration of serotonin as did those of normals. On the other hand, animals in which reserpine was given 2 hours before iproniazid showed a marked decline in brain serotonin.

The pharmacologic effects obtained when reserpine was administered in the presence of iproniazid were compared with the effects that followed administration of 0.1 to 0.2 mg/kg of LSD which has been shown to have no serotonin-releasing activity(4). Five animals given the hallucinogenic compound exhibited sympathomimetic responses including excitation, mydriasis, exophthalmus, and piloerection that could not be distinguished from those observed following administration of reserpine to animals pretreated with iproniazid. The effects induced by LSD could be overcome by the administration of either chlorpromazine (5 mg/kg) or reserpine (5 mg/kg).

The effects of reserpine and chlorpromazine were also determined in 6 animals grossly excited by administration of iproniazid followed by reserpine. Administration to 3 of these animals of another dose of 5 mg/kg of reserpine failed to reverse the excitation. On the other hand, when 5 mg/kg of chlorpromazine was given to the other 3 animals they became rapidly sedated.

The actions of the 2 tranquilizing agents were also compared in rabbits with an excess of free serotonin in the brain produced by the intravenous administration of 100 mg/kg of 5-hydroxytryptophan. As shown by Bogdanski *et al.*, this compound rapidly penetrates brain where it is decarboxylated to form serotonin, and, after large doses, induces excitement and sympathomimetic effects during the period that serotonin is being formed. Administration of 5 mg/kg of reserpine to rabbits pretreated with 5-hydroxytryptophan failed to reverse the excitation or the sympathomimetic effects but these signs were quickly reversed by chlorpromazine.

*Discussion.* Administration of reserpine to rabbits pretreated with iproniazid, an inhibitor of serotonin metabolism, causes excitatory and sympathomimetic effects typical of LSD, rather than sedation and parasympathetic predominance seen when reserpine is given alone. Furthermore, reserpine in the presence of iproniazid does not cause the precipitous decline in brain serotonin level seen after administration of reserpine alone. The reversal of the effects of reserpine is not due simply to the combination of drugs, for changing the order of administration results in the usual pharmacologic effects of reserpine and the usual decline in brain serotonin.

Excitation and other LSD-like responses can also be induced in animals by the administration of high doses of 5-hydroxytryptophan, which enters the brain and is there decarboxylated to form considerable amounts of serotonin. The effects of 5-hydroxytryptophan have been shown to be especially pronounced when the destruction of serotonin has been blocked by iproniazid. Thus, it is possible that the bizarre effects of the combination of iproniazid and reserpine are due to serotonin which has been changed by

reserpine from a bound to a free form but which is now protected from degradation by the presence of iproniazid.

It would appear that the usual parasympathomimetic effects of reserpine are associated with a low concentration of free serotonin in brain, whereas sympathetic predominance is associated with a high concentration of the free indole. This apparent difference in the behavior of serotonin, depending on whether it is present in low or in high concentration, is reminiscent of the behavior of acetylcholine, which in high concentration blocks its own action at peripheral ganglia(5). The findings with serotonin are not inconsistent with the concept that serotonin functions as a neurohumoral agent in the parasympathetic division of the central autonomic system. The blockade of central parasympathetic centers could unmask the antagonistic sympathetic centers and result in sympathetic predominance.

An important observation is that reserpine can overcome the excitation and sympathetic predominance induced by LSD but does not block similar responses resulting from a high concentration of free serotonin in brain, whether the free serotonin is produced by administration of its precursor, 5-hydroxytryptophan, or by the combination of iproniazid and reserpine. In contrast, chlorpromazine, which does not release brain serotonin (4), readily reverses the effects of both LSD and a high concentration of free serotonin, suggesting that although the two tranquilizing agents induce a number of similar pharmacologic responses, they do so by different mechanisms. It is possible that chlorpromazine produces its pharmacologic effects by direct blockade of central sympathetic centers.

*Summary.* 1. Administration of reserpine to rabbits pretreated with the monoamine oxidase inhibitor, iproniazid, causes excitation and sympathomimetic effects similar to those observed after administration of LSD or high doses of 5-hydroxytryptophan. These effects are associated with the presence of a high concentration of free serotonin in the brain. The observations are consistent with the concept that serotonin is normally bound in an inactive form which serves as the precursor form of a neurohumoral agent. 2. Although both chlorpromazine and reserpine reverse the effects of LSD, only chlorpromazine blocks the effects of free serotonin which result from administration of 5-hydroxytryptophan or the combination of iproniazid and reserpine. This suggests that the two tranquilizing drugs act by different mechanisms.

Iproniazid (Marsilid) was kindly supplied by Hoffmann-LaRoche; reserpine phosphate by Ciba Pharmaceutical Products, and lysergic acid diethylamide (LSD) by Sandoz Laboratories.

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Received December 4, 1956. P.S.E.B.M., 1957, v94.

# Delayed Hypersensitivity *in vitro*. I. Effects of Tuberculoproteins on Tissue from Sensitive Guinea Pigs.* (22969)

J. Darrell Shea and Herbert R. Morgan
*Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.*

In early experimentation on delayed hypersensitivity *in vitro,* Rich and Lewis(1,2) showed that tuberculin displayed a cytotoxic action for tissues from tuberculous guinea pigs which demonstrated positive skin tests with Old Tuberculin (O.T.). Since the introduction of Robert Koch's filtrate of heated broth culture of tubercle bacilli or O.T. as skin testing agent in *Mycobacterium tuberculosis* infection, many tuberculoproteins have been prepared for skin tests. Seibert(3) introduced a purified protein derivative of tuberculin (PPD) which has had wide acceptance. Subsequently, she isolated 3 tuberculoprotein fractions designated A, B, and C(4). Pangborn and Birkhaug(5) prepared a purified tuberculin fraction labeled T(NY) and believe it to be more sensitive than PPD in skin testing.

Using tissue culture technics, a study was undertaken to compare *in vitro* cytotoxic effects of 3 of the above-mentioned tuberculoproteins—T(NY), PPD, and C.

*Materials and methods.* Albino guinea pigs were sensitized by subcutaneous injection of 10 mg and intraperitoneal injection of 20 mg of a suspension of *Mycobacterium tuberculosis* bovine strain (BCG). Five weeks later the animals were skin tested for hypersensitivity by intradermal injection of 0.1 ml of 1/10 dilution of O.T. An acceptable positive reaction was characterized at 48 hours by erythema and marked induration, usually with a center of necrosis. The tissue culture method was the same as that outlined by Gangarosa *et al.*(6). The spleen was removed aseptically from freshly killed guinea pig and minced with corneal scissors in small test tube with Hanks' balanced salt solution (BSS)(7). Fragments about 1 $mm^2$ were placed on rooster plasma coagulum in T flasks (Kontes Glass Co.). Two ml of medium were added to each flask which was sealed with silicone stopper and incubated at 37°C. In these experiments the medium consisted of BSS (60%), beef serum ultrafiltrate (30%), and guinea pig serum (10%). The guinea pig serum was heated at 56°C for 30 minutes before adding to medium. Penicillin and streptomycin were added in concentrations of 75 units/ml and 100 γ/ml, respectively, to control bacterial contamination. An average of 48 fragments per test group were planted. Quantitative and qualitative evaluation of cytotoxic activity on splenic macrophages was made in the same manner as described by Gangarosa *et al.*(6) by observing and counting the number of macrophages seen to migrate from the fragment for 2 to 5 days. All tuberculoproteins were added to tissue cultures 24 hours after planting. The PPD, lot #95877, was supplied by Sharp and Dohme, Inc. Dr. Mary C. Pangborn of N. Y. State Department of Health, Albany, kindly supplied T(NY) protein and C protein.

*Results.* The possibility of nonspecific toxicity of the 3 tuberculoproteins for splenic macrophages was eliminated by results of a series of experiments with splenic tissue from normal tuberculin-negative guinea pigs. In 4 experiments, the migration of macrophages was not inhibited by these tuberculoproteins at concentrations of 50 γ/ml (Fig. 1). The macrophages in all flasks appeared healthy with many pseudopodia, clear cytoplasm, and large, distinct nuclei.

Addition of tuberculoprotein to splenic tissue from tuberculin-positive guinea pig resulted in definite toxic changes, including alteration of cellular morphology such as rounding up of the cell with loss of pseudopodia, coarse granularity of cytoplasm, and darkening of nucleus. No such changes were observed in control flasks to which no tuberculoprotein was added.

* Supported by a research grand from Eli Lilly Co., Indianapolis.

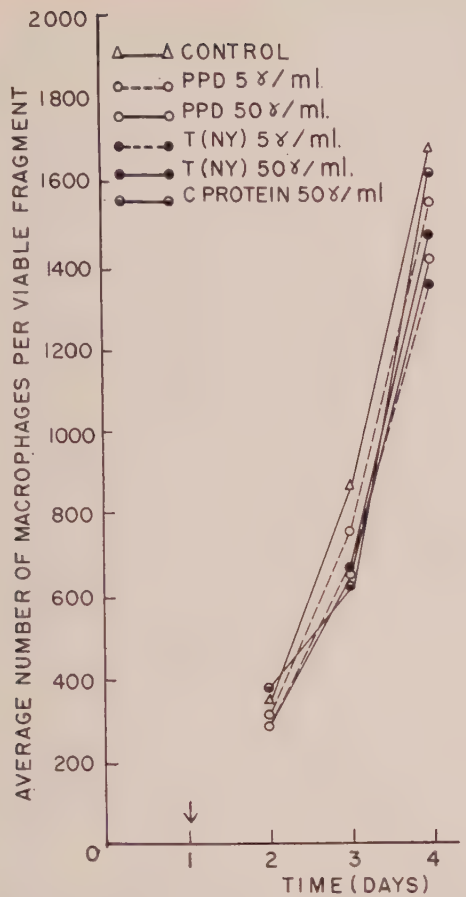


FIG. 1. Response of splenic macrophages from normal tuberculin-negative guinea pig to 3 tuberculoproteins, PPD, T(NY), and C.

Quantitative evidence for toxic effects of tuberculoproteins was found in marked inhibition of cellular migration. Daily cell counts on each of 3 successive days showed that both 5 and 50 γ/ml concentrations of PPD, T(NY), and C-protein inhibited the migration of macrophages from spleen fragments. All 3 tuberculoproteins produced the same degree of inhibition at both concentrations.

Anticipating a threshold for cytotoxicity at lower levels, 1 γ/ml was used. As shown in Fig. 2, PPD, T(NY), and C-protein were cytotoxic at concentration of 1 γ/ml. The degree of cellular inhibition by the tuberculoproteins at this low concentration is less marked than at 5 or 50 γ/ml, but all 3 proteins display the same intensity of cellular toxicity. Qualitatively, the cells revealed effects similar to those described in the previous experiment.

The absence of a threshold necessitated further dilution. The lower concentration of each protein in the protocol was changed to 0.1 γ/ml. Fig. 3 demonstrates that there is no significant difference between cell counts of test groups and the control group. Qualitatively migrating macrophages in cell test flasks were indistinguishable from control group cells and all appeared normal and healthy.

Not only did the 3 tuberculoproteins show the same activity on a dry-weight basis, but chemical analyses indicated that their nitrogen content was so similar that their effects were almost identical when expressed in terms of nitrogen content which was 16.3% for PPD(3), 14.1% for T(NY), and 14%

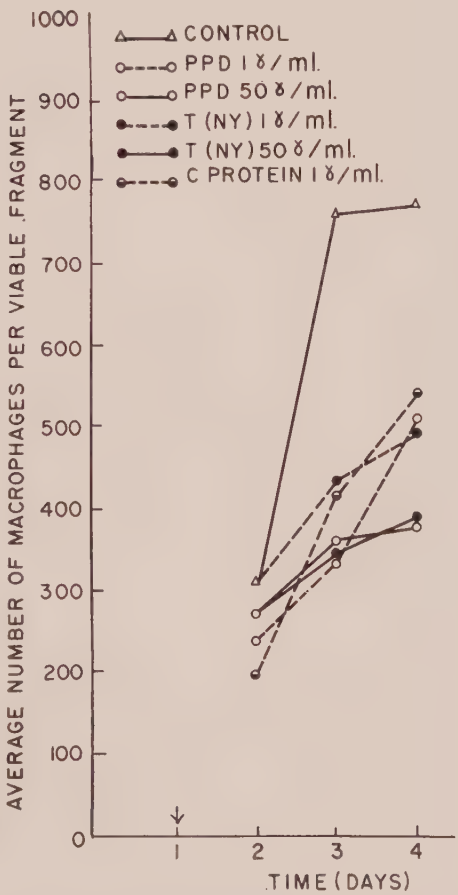


FIG. 2. Response of splenic macrophages from tuberculin-positive guinea pig to the tuberculoproteins PPD, T(NY), and C.

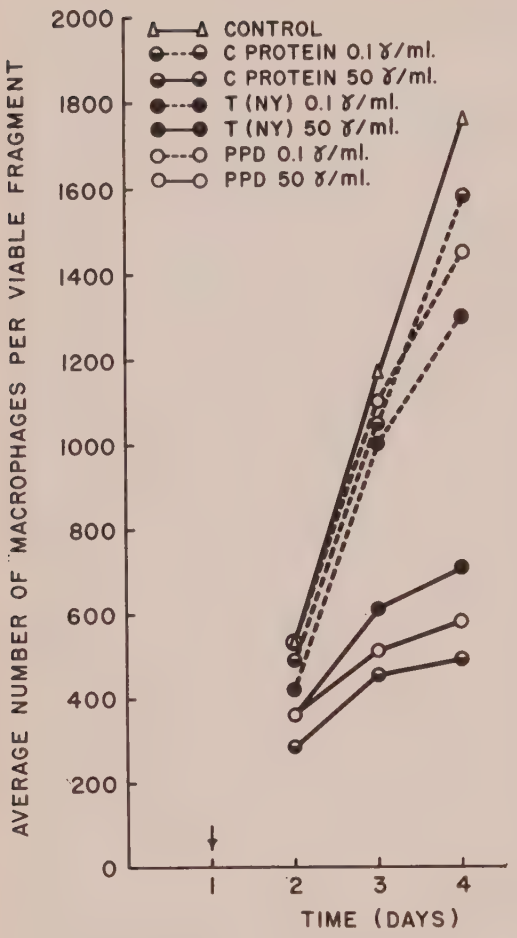


FIG. 3. Response of splenic macrophages from tuberculin-positive guinea pig to the tuberculoproteins PPD, T(NY), and C.

for C† tuberculoproteins, respectively.

No attempt was made to sharpen the end point of cytotoxicity by conducting experiments with tuberculoprotein concentrations between 1 and 0.1 γ/ml because the former concentration showed only moderate cytotoxic effects.

In an effort to compare the *in vivo* activities of these 3 tuberculoproteins with their *in vitro* effects, a series of skin tests was made on BCG-sensitized guinea pigs. All tuberculoproteins were used in final concentration of 100 γ in 0.1 ml which was injected into the skin of these guinea pigs. In no instance was a reaction to O.T. (1/1000) observed. There were no significant differences between the dermal reactions to the 3 tuberculoproteins—PPD, T(NY), and C.

*Discussion.* The experimental results reported here indicate that there is no significant difference in *in vitro* cytotoxic activity of the 3 tuberculoproteins at any of the concentrations studied, based either on dry weight or nitrogen content. All 3 tuberculoproteins are ineffective at 0.1 γ/ml.

It is interesting that it has been possible to demonstrate active cytotoxicity and cellular inhibition by these 3 tuberculoproteins at low concentrations of 1 γ/ml which is a much smaller quantity than has been used in similar experiments(1,2,6).

One might hypothesize that all of these tuberculoproteins contain one or more common constituents or configurations which elicit this toxic response. To determine the nature of this hypothetical active principle, it will be necessary to further purify these tuberculoproteins and to determine their chemical nature.

*Summary.* 1. The cytotoxicity of 3 tuberculoproteins, PPD, T(NY), and C, for tuberculin-sensitive macrophages has been compared in tissue culture. All 3 tuberculoproteins had cytotoxic properties *in vitro*. No differences were observed between *in vitro* effects of each of the tuberculoproteins at any one concentration. 2. Each tuberculoprotein had a definite observable cytotoxic and inhibitory action on tuberculin-sensitive splenic macrophages at low concentration of 1 γ/ml, but none at 0.1 γ/ml. 3. Skin test reactions in sensitized guinea pigs to the 3 tuberculoproteins gave similar results.

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Received December 13, 1956. P.S.E.B.M., 1957, v94.

# Somatotropic Activity of U. S. Pharmacopeia Growth Hormone Reference Standard in Alkali or Acid.* (22970)

E. A. Lazo-Wasem and Claire E. Graham
(With technical assistance of Joan B. Michalski)
*The Wilson Laboratories, Chicago, Ill.*

A United States Pharmacopeia (USP) Growth Hormone Reference Standard was released within the last year. One unit is defined as the activity of 1 mg of the Standard (1). Since the Standard was prepared by the U. S. Pharmacopeia as a service to those interested in it, and not because it is required for any pharmacopeial preparation, no specific information was made available regarding choice of assay methods or injection vehicles. It is our purpose to report 3-fold differences in potency as a result of dissolving growth hormone preparations in alkaline or acid solution.

*Methods.* Assays for somatotropic activity were made by the 10-day growth test in hypophysectomized, male, albino, 100 g rats. The animals were received from Hormone Assay Laboratories and were fed a diet containing 25% casein.† Plateaued rats failing to gain or lose over 5 g during the third postoperative week were selected for assay. The rats were distributed at random into groups of 5 to 7. Injections were made subcutaneously, 0.5 cc/rat/day, for 10 days. Final body weights were determined on the day after last injection. A comparison of the USP Standard against itself when injected in alkaline or acid solution was made by suspending 10 mg in distilled water and adding enough alkali or acid to dissolve. Final dilution was made with distilled water. The pH of injected solutions was pH 3.5 or pH 9.5. This experiment was also done with a pork pituitary preparation, STH 8, prepared at The Wilson Laboratories by the method of Raben and Westermeyer(3). The somatotropic potency of STH 8 was standardized against the USP Standard. In tests 1 and 2, 10 mg of each preparation were suspended in 8 ml of 0.9% saline, 3 drops of 0.1 N NaOH added, and diluted to 10 ml with saline. Further dilution to injected concentrations was also made with saline. The injected solutions had a pH range of 8.0-9.5. Identical procedure was followed in test 3, except that 0.5% phenol was used as diluent instead of saline. In tests 4 and 5, 10 mg each of STH 8 and USP Standard were dissolved in 0.5 ml of 0.05 N HCl, immediately diluted to 10 ml, and then to injected concentrations with 0.5% phenol. The pH of final solutions was between 3.5 and 4.0. The final solutions in all cases were refrigerated during the 10-day test period, except for one hour each day when they were brought to room temperature to make the injections. Since after the first day of refrigeration, the solutions made with alkali became turbid in various degrees, all solutions were vigorously shaken before injection. The acidified solutions remained clear during the course of the assay.

*Results.* The results of comparing each preparation against itself when injected in alkaline or acid solution are presented in Table I. These data show that the USP Standard shows a much smaller effect when injected in acid. On the other hand the activity of STH 8 in alkaline or acid media is the same.

TABLE I. Somatotropic Effect of USP Growth Hormone Reference Standard and STH 8, Pork Preparation Obtained by Method of Raben, Injected in Alkaline or Acid Solution to Hypophysectomized, Male, 100 g Rats (10-Day Test). Dose: 25 μg/day.

| Somatotropic preparation | Mean gain in g ± S.E. | | Statistical diff.,* alkaline *vs* acid |
|---|---|---|---|
| | Alkaline sol. | Acid sol. | |
| USP Std. | 17.6 ± 1.1 | 10.8 ± 1.8 | Signif. at $P_{.05}$ |
| STH 8 | 11.6 ± 1.2 | 12.2 ± .5 | Not signif. |

* Calculated by Fisher's "t" test.

* The authors acknowledge the interest and helpful criticisms of Dr. S. W. Hier.

† The composition of the diet in percent was: casein 25, milk powder 10, yeast 10, desiccated liver 3, salts IV(2) 2, sucrose 45 and corn oil 5.

TABLE II. Somatotropic Potency of STH 8, Pork Preparation Obtained by Method of Raben, when Assayed against the USP Growth Hormone Reference Standard (1 Unit = 1 mg) in Hypophysectomized, Male, 100 g Rats (10-Day Test).

| Test No. | STH 8 Dose, μg/day | STH 8 Gain, g/day | USP standard Dose, μg/day | USP standard Gain, g/day | pH of vehicle | Potency, USP units per mg | 95% confidence limits | Index of precision |
|---|---|---|---|---|---|---|---|---|
| 1 | 20 | .65 | 15 | .73 | Alkaline | .32 | .20- .51 | .17 |
| | 80 | 1.10 | 60 | 1.92 | | | | |
| 2 | 40 | 1.37 | 20 | 1.59 | " | .32 | .21- .49 | .26 |
| | 120 | 1.76 | 60 | 2.17 | | | | |
| 3 | 40 | .80 | 20 | 1.30 | " | .18 | .12- .28 | .19 |
| | 120 | 1.35 | 60 | 1.94 | | | | |
| 4 | 30 | 1.08 | 20 | .68 | Acid | 1.05 | .68-1.62 | .21 |
| | 90 | 2.02 | 60 | 1.62 | | | | |
| 5 | 40 | 1.40 | 30 | .90 | " | .83 | .52-1.33 | .30 |
| | 120 | 1.68 | 90 | 2.05 | | | | |

The somatotropic potency of STH 8 in terms of the USP Standard is shown in Table II. The pork preparation, STH 8, exhibited an activity slightly less than one-third that of the USP Standard (0.32, 0.32 and 0.18 unit/mg), when an alkaline solution was used, but when an acid solution was utilized the activity of STH 8 was equivalent to that of the USP Standard (1.05 and 0.83 USP unit/mg).

*Discussion.* The data in Table I indicate that the USP material appears less active in acidic than in basic solution, while STH 8, the pork preparation obtained by the Raben method, produces equivalent responses on both sides of neutrality. This method employs glacial acetic acid extraction, while the USP Growth Hormone Standard was prepared from beef according to the method of Wilhelmi, Fishman and Russell(4), which employs alkaline extraction. The two preparations reported here, therefore, differ in two factors: the method of preparation and the animal source, and their differential reactivity in alkali or acid could be due to either factor. The decreased stability in acid of the USP material reported here, is in agreement with work by Li and Papkoff(5) who reported definite destruction of activity with accompanying changes in electrophoretic behavior when alkali extracted beef growth hormone was dissolved in 0.1 N acetic acid and maintained at 25°C for 24 hours.

*Conclusions.* 1. The somatotropic activity of a pork pituitary preparation, obtained by the method of Raben was compared with that of the newly released USP Growth Hormone Reference Standard. Some results were dependent on the pH of the injection vehicle. When alkaline solutions were used, the Raben preparation was about one-third as active as the USP material, but when injections were made in an acid vehicle both preparations exhibited equi-potent activity. 2. When somatotropic preparations are assayed against the USP Growth Hormone Reference Standard an alkaline solution must be used, since the USP material shows decreased activity when dissolved in acid.

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Received December 14, 1956. P.S.E.B.M., 1957, v94.

# Experimental Hypervitaminosis D: Hypercalcemia, Hypermucoproteinemia, and Metastatic Calicification. (22971)

Reuben Eisenstein and William A. Groff
(Introduced by J. H. Wills)
*Pathology Branch, Directorate of Medical Research, Army Chemical Center, Md.*

Administration of massive doses of vit. D results in a disease, hypervitaminosis D, characterized by increased absorption of calcium and phosphorus, hypercalcemia, high urinary excretion of calcium, resorption of bone, and metastatic calcification(1). Engel(2) found that rats given large doses of parathyroid hormone showed a rise in blood levels of a mucoprotein called seromucoid (3) and an increase in concentration of soluble seromucoid in bone. He also observed alterations in staining characteristics of bone which suggested a depolymerization of bone matrix, and described deposition of calcium salts in renal tubular casts which contained polysaccharide. He suggested that parathyroid hormone induced a depolymerization of bone matrix so that mucoprotein components of the matrix leaked into the body fluids.

The present study was undertaken to determine whether hypervitaminosis D is associated with a change in seromucoid level of blood. The relationship of polysaccharide matrices to site of deposition of metastatic calcification was also studied.

*Methods.* Thirteen female white rats of the Wistar strain weighing 90-140 g were fed a diet of commercial Purina chow and given water *ad libitum*. The animals were injected intraperitoneally with 200,000 units of vit. $D_2$ in cottonseed oil (from Nutritional Biochemicals Co., Cleveland) daily for 8 days. Fourteen control animals were injected intraperitoneally daily with equal volumes of cottonseed oil. Untreated rats were also studied. Rats with hypervitaminosis D lost 15-30% of their body weight; therefore 8 untreated rats were starved for 5 days, during which they lost 40% of their body weight, and were examined as additional controls. Another group of 8 rats received 100,000 units of vit. $D_2$ for 3 days, with 8 animals receiving an equal volume of cottonseed oil as controls. At termination of dosage schedules, the rats were anesthetized with ether and exsanguinated by aortic puncture. Blood serum was separated and frozen in dry ice. Serum levels of calcium were determined by the method of Rehell(4). The levels of seromucoid were measured by the method of Winzler(3) and are expressed as mg of galactose-mannose/100 ml of serum. Seromucoid is the name given by Winzler to a mucoprotein which has been well characterized and found to be rich in galactose and mannose. The blood level of this compound increases in many conditions in which there is alteration of connective tissue, *e.g.*, infections and metastatic tumors(3), scurvy(5), and hypervitaminosis A(6). Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-7 $\mu$. Histologic sections were prepared with hematoxylin-eosin, with toluidine blue and with the periodic acid-Schiff method for polysaccharides before and after digestion with salivary amylase. Persistence of red color of periodic acid-Schiff reaction after digestion with amylase indicates that the color is not due to glycogen. Metachromasia in toluidine blue preparations demonstrates acid mucopolysaccharides. Some sections were colored by Giemsa's

TABLE I. Seromucoid Levels in Hypervitaminosis D.

| | No. of animals | Mean | Stand. dev. | Probability |
|---|---|---|---|---|
| No treatment | 18 | 9.8 | 2.3 | .012 * |
| Starved 5 days | 9 | 7.9 | 1.1 | |
| Cottonseed oil, 8 days | 14 | 13.0 | 6.35 | .0019* |
| Vit. D, 200,000 units, 8 days | 13 | 20.8 | 6.16 | |
| Cottonseed oil, 3 days | 8 | 11.3 | 2.31 | .0071 |
| Vit. D, 100,000 units, 3 days | 8 | 14.9 | 2.81 | |

* Approximately.

method, by alizarin red, and by Masson's trichrome method. Alizarin red demonstrates calcium.

*Results. Blood chemistry.* Serum calcium rose from control levels of 9-11 mg% to levels of 14-18.5 mg% as measured on the third and eighth day of treatment with vit. $D_2$. Calcium levels did not change in animals receiving cottonseed oil. Seromucoid levels are tabulated in Table I together with a statistical analysis. One sided "t" tests were performed.

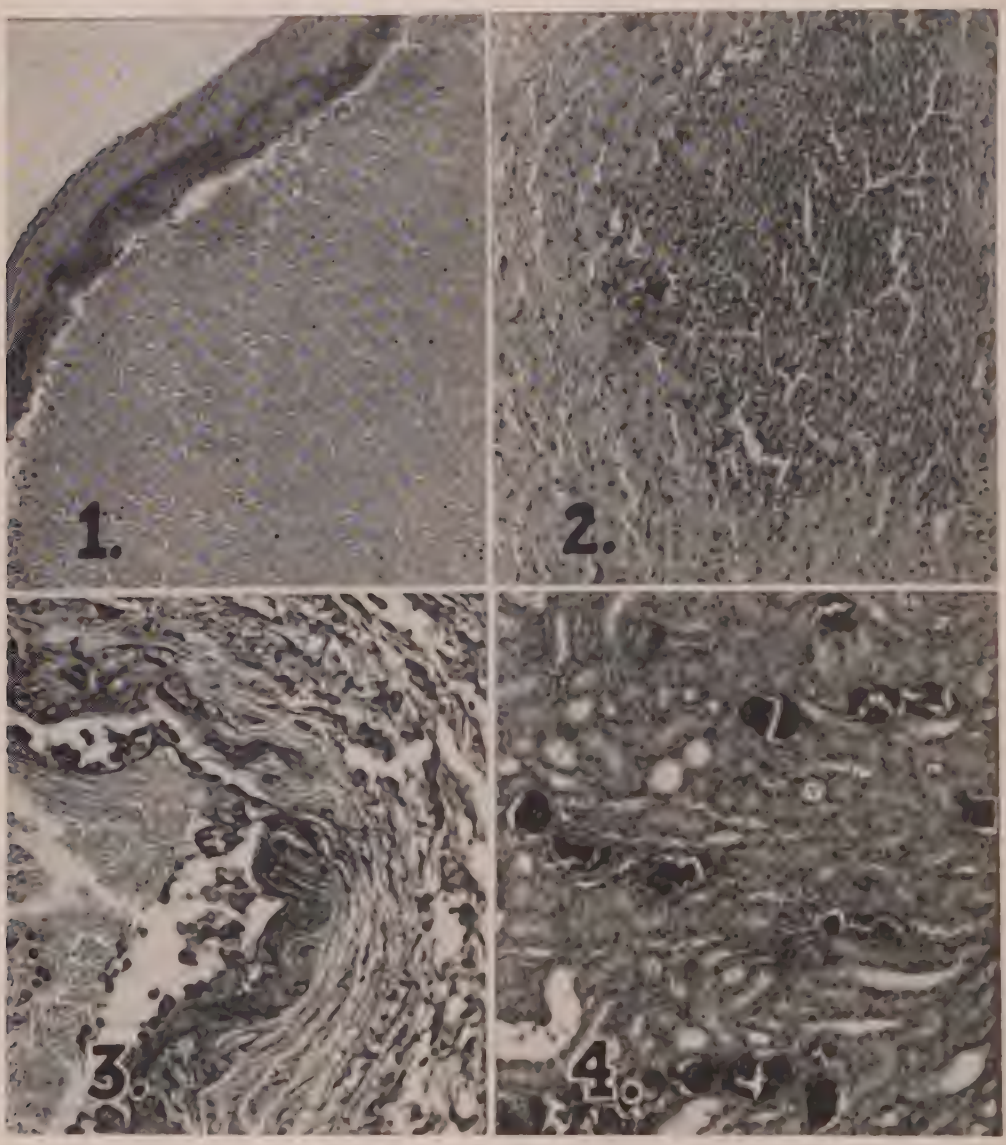


FIG. 1. Aorta of rat with hypervitaminosis D showing calcification of media. Hematoxylin-eosin stain ×140.

FIG. 2. Heart of rat with hypervitaminosis D showing myocarditis unassociated with calcific deposits. Hematoxylin-eosin stain ×140.

FIG. 3. Aorta of hypervitaminotic rat showing elevation of endothelium with collection of mononuclear cells beneath it, and accumulation of amorphous metachromatic material in interfibrillar spaces of the media. Toluidine blue stain ×400.

FIG. 4. Hypervitaminotic rat kidney with calcified casts in tubular lumina. Hematoxylin-eosin stain ×140.

It can be seen that seromucoid levels are higher in rats injected with vit. $D_2$ than in those injected with cottonseed oil.

The histological changes described below were found only in those animals which had received 200,000 units of vit. $D_2$ for 8 days, and were, in general, similar to those which have been described previously by Ham(7). No such changes were observed in animals injected with 100,000 units of vit. $D_2$ for 3 days or in those which had received only cottonseed oil.

*Kidney.* Tubular casts of 2 types were found; brightly eosinophilic casts and others which were calcified. Both types were bright red in periodic acid-Schiff preparations before and after digestion with salivary amylase. Calcified casts were metachromatic, while uncalcified casts were rarely found to be metachromatic.

*Heart*: A severe myocarditis was seen. It was characterized by destruction of cardiac muscle fibers, and an inflammatory exudate consisting primarily of large mononuclear cells, with some lymphocytes and Anitschkow myocytes. Calcareous deposits were present in many of the inflammatory foci, but many other foci were free of histologically identifiable calcium. In the areas of inflammation, there was an accumulation of extra-cellular material which was colored red in periodic acid-Schiff preparations and was metachromatic. Many branches of the coronary arteries showed calcific deposits in the media and accumulation of amorphous material in all coats of the vessels. Both calcific deposits and amorphous material were periodic acid-Schiff positive and metachromatic.

*Aorta*: The arch of the aorta showed 3 types of histological alterations; elevation of endothelium with accumulation of mononuclear cells beneath it, calcification of media and elastic interna, and collection of amorphous interfibrillary material in the media. Calcium was encrusted upon the elastic fibers. Again, at sites of calcareous deposition, there was an accumulation of metachromatic, periodic acid-Schiff positive material. Amorphous interfibrillary material was also metachromatic and periodic acid-Schiff positive.

*Interpretation of results*: Since the work of Harris and Innes(1), there has been abundant evidence that administration of massive doses of vit. D results in dissolution of bone (10,11). In parathyroid hormone poisoning, there is an increase in blood level of seromucoid which apparently has its origin in the bone matrix(2). Our experiments have demonstrated 3 changes in rats with hypervitaminosis D: (1) a rise in level of serum calcium, (2) an increase in serum seromucoid, and (3) deposition of calcium in tissues in a matrix composed at least partially of an unidentified polysaccharide. It would seem reasonable, then, to suggest that administration of massive doses of vit. $D_2$ results in release of bone minerals and organic matrix into the general circulation with their subsequent deposition in the soft tissues.

Tissue stains in our experiments demonstrate only the presence of polysaccharides in general. The polysaccharides of connective tissue consist of a variety of molecular species(12). In dissolution of bone many of these must be degraded or released from their bound state in the tissue, so it would seem unlikely that seromucoid itself is directly involved in deposition of calcium. The calcium-binding components of the matrix may be other compounds not measured here, perhaps chondroitin sulfates.

Rubin and Howard(8) found that calcification of soft tissues in a variety of locations and pathologic states occurred in a matrix of chondroitin sulfate, as revealed by histochemical evidence. However, Hass(9) pointed out that pathologic calcification occurs in a variety of matrices, some of which seem to be free of chondroitin sulfate.

*Summary.* 1. Rats given massive doses of vit. $D_2$ show increased serum levels of calcium and seromucoid (a mucoprotein), calcareous deposition in the heart, kidneys, and blood vessels, and accumulation of polysaccharides in the same locations. Myocarditis, unassociated with deposition of calcium as assessed histologically, was observed. 2. Calcium deposits occur in a matrix containing acid mucopolysaccharides.

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Received December 18, 1956. P.S.E.B.M., 1957, v94.

## Sodium Retaining Activity of 19-Nor-Steroids in Adrenalectomized Rats. (22972)

C. M. KAGAWA AND C. G. VAN ARMAN

*Biological Research, G. D. Searle & Co., Chicago, Ill.*

Small changes in the structure of steroids cause large differences in sodium retaining activity. Aldosterone, for example, possesses about 25 times the potency of desoxycorticosterone(1-3). The 9$\alpha$-halo-analogs(1,4-6) of cortisone and hydrocortisone and their 6-dehydro-modifications(7) are stronger sodium retainers than the parent compounds. Hydrocortisone is only weakly active, but its 2-methyl- and 2-methyl-9$\alpha$-halo-derivatives (8,9) are highly potent. Large differences in sodium retention are also seen with removal of the methyl group at carbon 10 (the 19-nor-steroids).

The present report summarizes our results in adrenalectomized rats with a series of these steroids, synthesized by Colton and associates(10,11) of our laboratories.

*Materials and methods.* The method of bioassay has been described in detail(12). Solutions of steroids in corn oil are injected subcutaneously into adrenalectomized rats with a saline load. A 2-hour sample of urine is collected in the bladder and analyzed for sodium. Amounts of 2 to 12 $\mu$g of desoxycorticosterone acetate (DCA) caused by this method a progressive retention of sodium. To minimize the day-to-day variation, one group of rats receiving DCA was used as a standard. An active response was defined arbitrarily as retention of sodium which was at least equal to that of 6 $\mu$g DCA. The probability is about 0.05 that an inactive steroid will elicit an equivalent or greater effect. Two steroids, 19-nor-desoxycorticosterone acetate and 19-nor-Reichstein's Compound S acetate, were compared separately with DCA in 4-point bioassays after several preliminary tests. Sodium concentrations were determined on the Beckman flame photometer for calculation of total excretion.

*Results.* The effects of nine 19-nor-steroids in adrenalectomized rats are summarized in Table I, together with data from simultaneous tests of 6 $\mu$g DCA. Sodium excretion with no DCA was 116 $\mu$Eq/rat in early tests with 111 rats.

With 19-nor-desoxycorticosterone acetate (I), doses of 1.5 to 100 $\mu$g elicited a marked retention of sodium. Results of a 4-point bioassay with 0.75 and 1.5 $\mu$g of the compound and 2 and 4 $\mu$g of DCA indicate that I is approximately 5.1 times as potent as DCA. The results of the test are illustrated in Fig. 1.

The data show that sodium retaining activity is decreased strongly by an hydroxyl group placed at carbon 11 or 17. Fig. 2

TABLE I. Effects of 19-Nor-Steroids on Sodium Excretion in Adrenalectomized Rats.

| Chemical name | 19-Nor-steroids | | | Desoxycorticosterone acetate (DCA) | | |
|---|---|---|---|---|---|---|
| | Dose, μg/rat | No. animals | Sodium excreted, μEq/rat | Dose, μg/rat | No. animals | Sodium excreted, μEq/rat |
| 3,20-Dioxo-19-norpregn-4-ene-21-ol-21-acetate (19-nor-desoxycorticosterone acetate) (I) | 100 | 4 | 10* | 6 | 3 | 56 |
| | 50 | 5 | 10* | 6 | 5 | 23 |
| | 6 | 5 | 12* | 6 | 5 | 23 |
| | 1.5 | 5 | 11* | 6 | 5 | 17 |
| | .75 | 5 | 56 | 6 | 5 | 48 |
| 3,20-Dioxo-19-norpregn-4-ene-17α,21-diol-21-acetate (19-nor-Reichstein's Compound S Acetate) (II) | 25 | 6 | 26* | 6 | 6 | 36 |
| | 8 | 5 | 56 | 6 | 5 | 27 |
| 3,20-Dioxo-19-norpregn-4-ene-11β,21-diol (19-nor-corticosterone) (III) | 200 | 6 | 10* | 6 | 6 | 60 |
| | 50 | 6 | 73 | 6 | 6 | 29 |
| 3,20-Dioxo-19-norpregn-4-ene-11β, 17α,21-triol (19-nor-hydrocortisone) (IV) | 100 | 4 | 11* | 6 | 3 | 56 |
| | 60 | 4 | 23* | 6 | 3 | 56 |
| | 25 | 4 | 10* | 6 | 5 | 27 |
| | 12 | 6 | 79 | 6 | 6 | 24 |
| 3-Oxo-19-norpregn-4-ene-17α,20Σ, 21-triol-20,21-diacetate (V) | 200 | 6 | 124 | 6 | 6 | 27 |
| 3-Oxo-19-norpregn-4-ene-17β,20Σ, 21-triol (VI) | 200 | 6 | 113 | 6 | 6 | 50 |
| 3-Oxo-19-norpregn-4,16,20-triene (VII) | 200 | 4 | 136 | 6 | 5 | 28 |
| 3-Oxo-17α-ethynyl-19-norandrost-4-ene-17β-ol (17α-ethynyl-19-nortestosterone) (VIII) | 200 | 4 | 77 | 6 | 6 | 27 |
| 3-Oxo-17α-ethyl-19-norandrost-4-ene-17β-ol (17α-ethyl-19-nortestosterone) (IX) | 200 | 8 | 147 | 6 | 8 | 48 |
| | 400 | 8 | 107 | 6 | 8 | 48 |

* Active response (P = 0.05 or less).

Sodium excretion with no DCA (corn oil alone) was 116 μEq/rat in early tests with 111 animals.

summarizes results of bioassay in which 9 and 18 μg doses of 19-nor-Reichstein's Compound S acetate (II) were run simultaneously with 3 and 6 μg DCA. A potency of 0.25 is found for II by this comparison. We find that if II is considered as a struc-

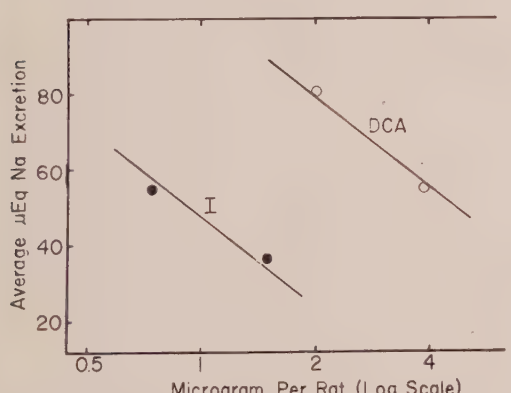


FIG. 1. Relative potencies of DCA and 19-nor-desoxycorticosterone acetate (I) in retention of sodium (DCA = 1, I = 5.1). Each point represents avg response of 5 animals.

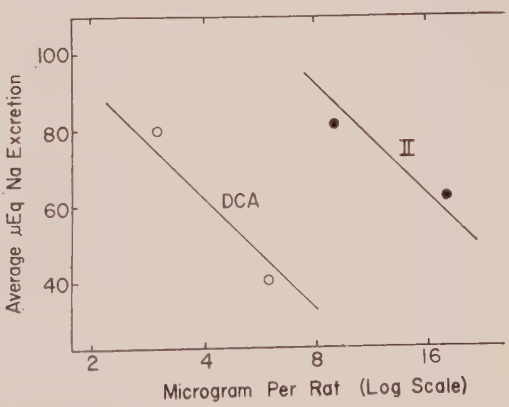


FIG. 2. Relative potencies of DCA and 19-nor-Reichstein's Compound S acetate (II) in the retention of sodium (DCA = 1, II = 0.25). Each point represents avg response of 5 animals.

turally modified 19-nor-desoxycorticosterone the hydroxyl group at carbon 17 reduces activity by 20-fold (5.1/0.25). Similarly, a loss of activity occurs when the same group is placed at carbon 11, as shown for 19-nor-corticosterone (III). Quantitative data for this compound are not available, but the effects with 50 and 200 μg doses suggest a potency of about 0.06 compared with DCA. An oxygen function at carbon 11, therefore, reduces activity of 19-nor-desoxycorticosterone by approximately 85-fold (5.1/0.06).

We find in 19-nor-hydrocortisone (IV) hydroxyl functions at both carbon 11 and 17. Definite salt activity is demonstrated with 25 to 100 μg, but not with a smaller dose. These results suggest a potency of about 0.25 times that of DCA, as discussed earlier for II.

The Table also summarizes data for 5 additional steroids, V, VI, VII, VIII and IX. Except for the 3-oxo-α, β-unsaturated system in ring A, these compounds show less structural resemblance to adrenocortical steroids, than those discussed earlier. Using 200 μg doses, an effect as strong as that of 6 μg DCA could not be seen. Likewise, no activity was found with 400 μg of IX.

*Discussion.* Our results demonstrate that removal of the methyl group at carbon 10 confers varying degrees of sodium retaining activity, depending upon specific structures of the steroids.

In our experience, 19-nor-desoxycorticosterone acetate shows 5.1 times the potency of DCA in sodium retention. Using the criterion of urinary sodium/potassium(13), this steroid has been reported by Sandoval, Miramontes, Rosenkranz, Djerassi and Sondheimer(14) to be approximately twice as active as desoxycorticosterone. Axelrod, Cates, Johnson and Luetscher(1) measured potencies of 1.9 and 1.5 times DCA using sodium and sodium/potassium responses, respectively. Altogether, these findings suggest increased activity for 19-nor-desoxycorticosterone, and in part do not appear consistent with the lack of survival activity reported by Ehrenstein (15).

The 19-nor-analogs appear to show stronger sodium retaining activity than the corresponding steroids with a methyl group at carbon 10. For example, 200 μg of Reichstein's Compound S produce an effect equivalent to about 8 μg DCA (*i.e.*, a potency of 0.04) (1,16). Its 19-nor-counterpart (II) is 0.25 times as potent as DCA by our measurements. Corticosterone is inactive at 10 to 400 μg doses according to the data of Johnson(16) and Marcus, Romanoff and Pincus(17). Results of our study show a potency of 0.06 (relative to DCA) for 19-nor-corticosterone (III). Likewise, while all observations on activity of hydrocortisone have proved negative(16,17), our studies show 19-nor-hydrocortisone (IV) to be 0.25 times as active as DCA.

The results with the last 5 steroids in Table I suggest that merely removing the methyl group at carbon 10 is not sufficient for increases in sodium retaining activity. Thus, structural and spatial alterations in the ketol side chain (V, VI, VIII and IX) and further changes in ring D (VII) do not give rise to activity. None of these steroids has as much activity as 6 μg DCA. It appears that a 19-nor-modification confers increasing activity only in steroids which show structurally a resemblance to the series of adrenocortical hormones.

*Summary.* 1. Sodium retaining activity of nine 19-nor-steroids was tested in adrenalectomized rats. 2. The 19-nor-analogs of desoxycorticosterone, Reichstein's Compound S, hydrocortisone and corticosterone showed 5.1, 0.25, 0.25 and 0.06 times the potency of desoxycorticosterone, respectively. 3. Sodium retention equal to that of 6 μg desoxycorticosterone was not detected in 5 other 19-nor-steroids, which showed less structural resemblance to adrenocortical steroids. 4. Our results indicate that varying degrees of activity will be found with removal of the methyl group at carbon 10, depending upon specific structure of the steroid.

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Received December 20, 1956. P.S.E.B.M., 1957, v94.

## Synthesis and Metabolism of Quinolinic Acid Ring Labeled with Tritium.* (22973)

L. V. HANKES AND I. H. SEGEL†
(Introduced by Donald D. Van Slyke)
*Medical Department, Brookhaven National Laboratory, Upton, N. Y.*

Since quinolinic acid was identified as a product of tryptophan metabolism in the rat (1), many experiments have been conducted to establish its role in tryptophan metabolism scheme(2). Isotope work has definitely proved that quinolinic acid was a product of tryptophan metabolism(3), and that 3-hydroxyanthranilic acid was converted to quinolinic acid in the rat(4). Various publications have suggested that in the sequence of reactions converting tryptophan to niacin, quinolinic acid functions as a side reaction product(5,6) and that quinolinic acid is not converted to nicotinic acid(7). Synthesis of quinolinic acid labeled in the ring with tritium has made it possible to obtain more dependable information regarding the role of quinolinic acid as an intermediary product in niacin formation. In our experiments labeled quinolinic acid was injected intraperitoneally into rats and labeled $N^1$-methylnicotinamide was isolated from the urine.

*Methods. Preparation of 4,5,6 tritium-labeled quinolinic acid.* The tritium ring labeled quinolinic acid was prepared by exposing a mixture of one gram of pure quinolinic acid (recrystallized 12 times) and 110 mg of lithium carbonate to a neutron flux of $1.8 \times 10^{12}$n/cm$^2$/sec for 48 hours. This method of tritium-recoil labeling is described by Rowland and Wolfgang(8). After exposure in the nuclear reactor, the quinolinic acid, which had turned from white to tan in color, indicating some decomposition, was dissolved in a small volume of water. This solution was adjusted to pH slightly below 10 with sodium hydroxide to remove the tritium from the carboxyl groups by salt formation. After concentrating the solution to dryness under vacuum and redissolving in water, the quinolinic acid was purified by column chromatography through the following steps: adsorption onto norite at pH of 1.4, elution of norite with 0.1 *N* $NH_4OH$, concentration of eluate to dryness, extraction of concentrate with acid methanol, and subse-

* This research was supported by Atomic Energy Com.

† Recipient of Brookhaven National Laboratory Undergraduate Summer Student Award, Summer 1956. Permanent address: Rensselaer Polytechnic Institute, Troy, N. Y.

TABLE I. Twenty-Four Hour Excretion of $N^1$methylnicotinamide and Quinolinic Acid after Injection of Ring Labeled Quinolinic Acid.

| Rat No. | | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Sp. activity of inj. Q-acid, μc/mMol | *(a)* | 23.29 | 23.29 | 23.29 | 23.29 |
| Quinolinic acid inj., mg | | 5 | 5 | 20 | 20 |
| *Idem* mMol | | .030 | .030 | .120 | .120 |
| Tritium inj., μc | *(b)* | .697 | .697 | 2.79 | 2.79 |
| *$N^1$methylnicotinamide* | | | | | |
| N-amide excreted, mg | | .235 | | .770 | |
| *Idem* mMol | *(c)* | .0017 | | .0056 | |
| Carrier added, mg/mg urinary N-amide | | 973 | | 346.3 | |
| Sp. activity of excreted N-amide, μc/mMol | *(d)* | 5.49 | | 1.78 | |
| Tritium excreted in N-amide | | | | | |
| $\mu c = c \times d$ | *(e)* | .0094 | | .0100 | |
| % of inj. $= \frac{100\ e}{b}$ | | 1.35 | | .36 | |
| Ratio, $\frac{\text{Sp. ac. of excreted N-amide}}{\text{Sp. ac. of inj. Q-acid}} = \frac{d}{a}$ | | .236 | | .076 | |
| *Quinolinic acid* | | | | | |
| Q-acid excreted, mg | | | 4.75 | | 13.49 |
| *Idem* mMol | *(f)* | | .028 | | .081 |
| Carrier added, mg/mg urinary Q-acid | | | 114 | | 40 |
| Sp. activity of excreted Q-acid, μc/mMol | *(g)* | | 23.95 | | 26.11 |
| Tritium excreted in Q-acid | | | | | |
| $\mu c = f \times g$ | *(h)* | | .68 | | 2.11 |
| % of inj. $= \frac{100\ h}{b}$ | | | 97.6 | | 75.5 |
| Ratio, $\frac{\text{Sp. ac. of excreted Q-acid}}{\text{Sp. ac. of inj. Q-acid}} = \frac{g}{a}$ | | | 1.028 | | 1.121 |

quent adsorption of the methanol extract onto an alumina column. The alumina column was eluted with 0.01 *N* $NH_4OH$. The eluate fraction of pH 8.8-9.2 was collected and then concentrated to dryness under vacuum. The crude quinolinic acid thus obtained was dissolved in 40% ethanol, treated with activated charcoal, filtered, and precipitated from solution by adjustment to pH 2 with concentrated hydrochloric acid. After several recrystallizations from small amounts of 50% ethanol, the material was paper chromatographed to check for purity. The labeled quinolinic acid gave a single component of the same Rf as commercial quinolinic acid. It had a specific activity of 306,750 counts/minute/mg of compound, when analyzed by gas counting procedure of Christman(9). *Animal experiments.* Male albino rats from Carworth Farms, weighing 280-380 g (raised on stock diet) were transferred to 9% casein-sucrose diet described earlier(10). After 5 weeks, the rats receiving this diet were housed in metabolism cages. Labeled quinolinic acid was administered intraperitoneally in 0.4 to 1.6 ml of 0.9% saline solution. One pair of rats received 5 mg, another pair received 20 mg of labeled quinolinic acid each. Twenty-four hour subsequent urines were collected. All urine samples were assayed for quinolinic acid and $N^1$methylnicotinamide. Quinolinic acid was determined by microbiological assay(11) and $N^1$methylnicotinamide was determined by a fluorimetric method using a Lumetron Fluorescence Meter, model 402-EF(12). With the aid of carrier quinolinic acid was isolated from 2 of the urines by previously described method(13). The $N^1$methylnicotinamide was isolated as the picrate by procedure previously described(14) with the use of carrier $N^1$methylnicotinamide prepared in this laboratory. (This $N^1$methylnicotinamide melted at 237° (corrected) and was shown by microbiological assay(11) to be free of nicotinic acid.) The purified quinolinic acid and $N^1$methylnicotinamide picrate from urines were analyzed for tritium content by the dry combustion and gas counting technic of

Christman(9).

*Conversion of quinolinic acid into N¹methylnicotinamide.* Table I shows that rats receiving 5 and 20 mg of labeled quinolinic acid excreted, respectively, 1.35 and 0.36% as labeled N¹methylnicotinamide. Activity ratios lead to the calculation that of the excreted amide 23.5 and 7.6%, respectively, in the 2 rats was derived from the injected quinolinic acid. The calculation is based on the assumption that in transformation from quinolinic to N¹methylnicotinamide none of the tritium atoms attached to carbons 4, 5, and 6 of the quinolinic acid is detached. If detachment occurred, the proportion of amide derived from quinolinic acid would be greater than that calculated above.

*Quinolinic acid excretion.* Quinolinic acid excreted by rats receiving 5 and 20 mg amounted to 95 and 68%, respectively, of the amounts injected. The specific activity was definitely greater in quinolinic acid excreted than in that injected. It appears possible that metabolic destruction of labeled quinolinic acid may have been less than of the unlabeled, permitting a larger proportion of the former to pass into the urine. The excess tritium was greater in excreted quinolinic acid of Rat No. 4, which also apparently metabolized a greater proportion, to judge from the smaller fraction excreted. It does not appear likely that tritium enrichment occurred during recrystallization of the quinolinic acid, since previous recrystallizations of labeled quinolinic acid did not change its activity.

*Summary.* 1. Quinolinic acid was labeled in the 4, 5, and 6 positions with tritium by the neutron recoil method. 2. Intraperitoneal injection of labeled quinolinic acid into rats was followed by excretion of labeled N¹methylnicotinamide in urine during the next 24 hours. Tritium contents of excreted N¹ methylnicotinamide in 2 experiments indicated that 7.6 and 23.6% originated from the injected labeled quinolinic acid. The evidence is definite that quinolinic acid can serve in the rat as a source of niacin. 3. Most of the injected quinolinic acid was excreted as such in the urine. Quinolinic acid isolated from urines of 2 rats had specific activities of 103 and 112% of injected quinolinic acid. The increase in specific activity suggests that tritium-labeled quinolinic acid may have been less rapidly metabolized than the unlabeled substance.

We wish to thank Rhoda Palter for her technical assistance.

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Received December 21, 1956. P.S.E.B.M., 1957, v94.

# Multiple Nature of Inhibitory Factor (Factor I) from Brain. (22974)

John A. Brockman, Jr. and Sherman L. Burson, Jr.
(Introduced by H. P. Broquist)
*Nutrition and Physiology Section, Research Division, American Cyanamid Co., Pearl River, N. Y.*

Florey(1) and Lissak(2) have described the inhibitory effects of a substance or substances present in mammalian brain and spinal cord, and the name Factor I(1) has been suggested for this principle. In preparing concentrates of Factor I, Florey used as assay for activity the stretch receptor preparation of the crayfish(3). He also indicated that Factor I imitated the action of the inhibitory neurons in crustaceans(1). Equipment was available to us to use the "slow" closer and opener systems(4) of the crayfish claw (*Cambarus virilis*)* as an assay. Although the preparations fatigue rapidly enough to make quantitative evaluation difficult, we found in preliminary experiments that dialyzable aqueous extracts of fresh rabbit and pig brain showed inhibitory activity in both these systems. The activity was not destroyed when extracts were heated at 100°C for 30 minutes in either 0.1 *N* hydrochloric acid or 0.1 *N* sodium hydroxide under nitrogen or oxygen. Activity was not removed from solution by Amberlite IR-45† (OH form) but was removed by Amberlite IR-120† (H form), from which it could be eluted with hydrochloric acid. Activity was lost when dried aqueous extract was treated with ethanolic hydrochloric acid, but was regenerated by heating with dilute aqueous hydrochloric acid. These results, which suggested that the activity was associated with a compound having both a basic group and an esterifiable acidic group, led us to prepare and study the dialyzable, amphoteric fraction of fresh brain.

*Methods.* Beef brains (85 kg) were collected as soon as possible after slaughter (*ca.* 15 to 30 minutes) and were frozen in dry ice and stored in freezer until use. The frozen brains were pulverized with dry ice, and the resulting powder was added to 100 l of boiling water at such a rate that boiling continued. After 10 minutes the mixture was filtered with the aid of 22.5 kg of Hyflo‡, and the filter cake was washed with 100 l of hot water. The combined extracts were concentrated *in vacuo* to 8 l which contained 2 kg of solids. This was placed in cellulose bags and dialyzed against 80 l of distilled water at room temperature for 44 hours. The dialysate was heated with 500 g of Norit A and filtered. The resulting solution, containing 0.98 kg of solids, was poured through a column of Amberlite IR-120† (H form), 23″ x 6″ diameter. The column was washed with water and eluted with 40 l of 3 *N* hydrochloric acid. The eluate was concentrated to 1 l, diluted with equal volume of alcohol, and the precipitated salts were filtered. The filtrate was concentrated, diluted with water, concentrated again to remove ethanol, diluted, and stirred with 1 kg of Amberlite IR-45† (OH form) to remove excess hydrochloric acid and increase the pH to 4-5. The resulting solution (4 l, 319 g of solids) was made basic (pH 9) with ammonium hydroxide, decolorized with Norit A, and poured through a column of Amberlite IRA-400† (Cl form), 29″ x 2½″ diameter, prewashed with 0.05 *N* ammonium hydroxide until effluent was basic (pH 9). The column was washed with water and eluted with 0.2 *N* hydrochloric acid until eluate was strongly acidic and no longer gave a precipitate with saturated phosphotungstic acid. The eluate was neutralized to pH 5 with Amberlite IR-45† (OH form) and concentrated for use in ion-exchange chromatography. The fraction contained 14 g of solids.

A 1.69-g. aliquot of this material was chromatographed on a 580 mm x 38 mm diameter column of Dowex 50-X12§, 200-400 mesh,

* Supplied by General Biological Supply House, Chicago, Ill.

† Rohm & Haas, Philadelphia, Pa.

‡ Filter aid of Johns Manville, Inc., New York City.

§ Dow Chemical Co., Midland, Mich.

using 1.5 *N* hydrochloric acid as developer. Fractions (10 ml) were collected with automatic fraction collector and were analyzed for nitrogen content by slight modification of the procedure of Johnson(5). Four well separated bands were observed with maximum nitrogen content in the tubes indicated: I, tube 106; II, tube 147; III, tube 160; and IV, tube 185. The distribution of nitrogen recovered in the 4 bands was 16.2, 0.9, 2.3, and 80.6% in bands I to IV respectively. When aliquots of the 4 bands were tested in the opener system of the crayfish claw, I and IV showed inhibition. Band I gave a strong ninhydrin test, and a preliminary paper chromatogram indicated that the substance might be aspartic or glutamic acid. Tests in the opener system showed that the unknown, aspartic acid, and glutamic acid each caused inhibition in the range 10 to 100 $\gamma$/ml depending on the particular claw, the frequency of stimulation and age of preparation. When compared in the same claw the unknown and glutamic acid usually appeared slightly more active than aspartic acid. All solutions were adjusted to pH 6.7 before testing.

Additional paper chromatograms confirmed that the unknown was not aspartic and was probably glutamic acid. Proof that the substance was glutamic acid was obtained by evaporation of band I to dryness and recrystallization of residue from 12 *N* hydrochloric acid to give colorless crystals, melting at 190.5 to 191.5°, $[\alpha]_D^{25} + 22.8°$, (*c*, 1.27, 6 *N* HCl), and neutralization equivalent, 90.9 (expected for *l*-glutamic hydrochloride: m.p., 189-191.5°(6); $[\alpha]_D^{22.4}$, 25.0° (free acid, 31.2°(7)); and neut. eq. 91.8).

Evaporation of an aliquot of band IV gave a crystalline residue which was shown to be mostly ammonium chloride by tests with Nessler's reagent and with chloroplatinic acid (8). The inhibitory activity of this material was much greater than that of ammonium chloride, and paper chromatograms indicated a second component which gave a purple color with ninhydrin. It seemed likely that the activity of band IV was due primarily to the presence of this other amino acid which was neither glutamic nor aspartic acid. The recent disclosure(9) that $\gamma$-aminobutyric acid has strong Factor I activity in the stretch receptor test, prompted us to make a paper chromatographic comparison of this amino acid with the amino acid in band IV. Mobilities of the substances were the same in 2 solvent systems. Therefore, the activity of band IV is undoubtedly due to the presence of $\gamma$-aminobutyric acid.

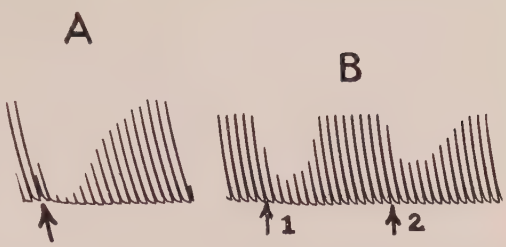


FIG. 1. Movement of dactylopodite of crayfish claws perfused at .5-.6 ml/min. on stimulation of motor axon for 3 sec. every 30 sec. with Grass Stimulator Model S4C giving square wave of .9 mSec. duration at frequency indicated below. At arrows the perfusion tube was momentarily squeezed shut and .5 ml of test solution was inj.: (A) Opener axon, 90 impulses/sec., glutamic acid at 100 $\gamma$/ml; (B) Slow closer axon, 30 impulses/sec.; 1, $\gamma$-aminobutyric acid at 100 $\gamma$/ml; 2, glutamic acid at 500 $\gamma$/ml.

The slow closer system of the crayfish claw was less sensitive than the opener system to glutamic and aspartic acids which gave inhibition at 100 to 1000 $\gamma$/ml depending on the particular claw, the frequency of stimulation, and age of preparation. Tests with $\gamma$-aminobutyric acid indicated that it was 5 to 10 times as active as glutamic acid. Typical kymograph tracings of the activities are shown in Fig. 1. A few experiments were conducted with the crayfish heart perfused with aerated crayfish solution(10). In the presence of 20 to 40 $\gamma$/ml of glutamic acid the heart rate became irregular and somewhat slower, and the contractions decreased considerably in amplitude, with the heart tending to remain relaxed. In the presence of *ca.* 100 $\gamma$/ml of aspartic acid the heart rate remained regular but became more rapid, and contractions decreased in amplitude, with the heart tending to remain in the contracted state. Neither glutamic acid (10 or 20 mg) nor $\gamma$-aminobutyric acid (10 or 20 mg) injected intraperitoneally into 20-g. Webster Swiss mice protected the animals from a lethal dose of strychnine (40 $\gamma$) injected 5 min-

utes later. It has been reported that the convulsive and lethal effects of strychnine in mice are prevented by injection of Factor I(11).

In view of the above results it is apparent that there is present in brain more than one substance having some of the activities ascribed to Factor I.

*Summary.* Extracts of fresh mammalian brain contained substances which inhibited the "slow" closer and opener systems of the crayfish claw. Glutamic acid was one of the substances. Aspartic acid and γ-aminobutyric acid were also active. Glutamic acid had an inhibitory effect and aspartic acid had a stimulatory effect on the crayfish heart.

The authors wish to express their appreciation to Mr. E. Starbird, Mr. P. F. Fabio, and Mr. R. E. Smith for technical assistance, and to Mr. W. Fulmor for optical rotation.

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Received December 26, 1956. P.S.E.B.M., 1957, v94.

## Production of Hypercholesteremia in the Rabbit by Infusion of Phosphatide or Neutral Fat.* (22975)

MEYER FRIEDMAN AND SANFORD O. BYERS
(With technical assistance of Clarence Omoto, Warren Hayashi, and Betty Tsai)
*Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.*

Recently we reported(1,2) that a rapid rise occurred in plasma cholesterol concentration of the rat, if a *sustained* rise in either its plasma phospholipid or triglyceride was induced initially by intravenous infusion of phosphatide or triglyceride. The mechanism responsible for the hypercholesteremic effect of a sustained hyperphospholipidemia or hypertriglyceridemia has not been determined but it has been observed(3) that a hypercholesteremia occurring after infusion of phosphatide takes place, surprisingly enough, in complete absence of the liver.

To determine whether this hypercholesteremic effect of experimentally induced and maintained hyperphospholipidemia or hypertriglyceridemia was applicable to other species, studies were made upon the rabbit.

* Aided by Grant from Life Insurance Medical Research Fund.

*Methods.* Healthy male rabbits weighing approximately 1500-1800 g and 6 to 8 weeks of age were used. A sustained hyperphospholipidemia was induced in six rabbits for 12 and in 5 rabbits for 24 hours by rapid intravenous administration of 20 ml of 5% phosphatides,† w/v, in 5% dextrose solution

† The phosphatide mixture was furnished by Chemurgy Division of Glidden Co., labeled Soya Lecithin, oil free, "RG" brand. Approximate composition as furnished by Glidden Co. as follows: Chemical lecithin, 29.5%; chemical cephalin, 29.5%; inositol phosphatides, 31.6%; sugar, sterol glucosides, etc., 5.3%; soybean oil, 3.1%; ether and insolubles and moisture, 1%. $CaCO_3$ is added in manufacture. Sterol content of this substance as measured by Liebermann-Burchard color reaction was 1.1%. Actual amount injected as 5% solution in our experiments therefore contained a negligible quantity of sterol (i.e. $<$ .6 mg/ml). Before use phosphatides were freed from $CaCO_3$.

TABLE I. Effect of Infusion of Phosphatide upon Plasma Cholesterol and Phospholipid.

| | Rabbits infused with phosphatide 12 hr | Rabbits infused with phosphatide 24 hr | Control rabbits* |
|---|---|---|---|
| No. | 6 | 5 | 5 |
| Avg wt (g) | 1760 (1400–1881) | 1682 (1400–1845) | 1500 (1340–1720) |
| Avg total amt phosphatide given (g) | 3.9 (1.0–5.8) | 2.6 (2.2–2.8) | — |
| Avg plasma cholesterol (mg/100 ml) | | | |
| Before inj. | 45 ( 27– 63) ± 4.4† | 69 ( 48– 96) ± 5.8 | 45 ( 33– 59) ± 4.8 |
| 12 hr after | 189 (110–294) ± 26.4 | — | 54 ( 48– 59) ± 4.2 |
| 24 " " | — | 153 (113–236) ± 19.2 | — |
| Avg plasma phospholipid (mg/100 ml) | | | |
| Before inj. | 119 ( 97–134) ± 5.8 | 129 ( 80–160) ± 6.4 | 110 ( 95–125) ± 6.3 |
| 12 hr after | 361 (158–496) ± 51.0 | — | 135 (115–159) ± 7.1 |
| 24 " " | — | 230 (171–394) ± 37.0 | — |

Fig. in parentheses give range of values.
* Infused 12 hr with dextrose (5%) solution. † S.E. of mean.

followed by continuous intravenous infusion of the same fluid at a rate of 4 ml/hour in rabbits infused for 12 hours and 1.5 to 2.0 ml/hour in rabbits infused for 24 hours. A sustained hypertriglyceridemia was induced in 8 rabbits by rapid intravenous injection of 20 ml of apricot oil (10%) emulsion‡ followed by continuous intravenous infusion of the same emulsion at 2 ml/hour for 10 hours. For control purposes 1) 5 rabbits were injected and infused with comparable volumes of dextrose solution alone; 2) 3 rabbits were injected and infused with dextrose (5%) and Tween (0.5%); 3) 3 rabbits were bled immediately after the initial injection of 20 ml of phosphatide and 4) 4 rabbits were bled immediately after the injection of 20 ml of the fat emulsion. Plasma samples were obtained before and at the end of the infusions. Those obtained from the rabbits infused with phosphatide were analyzed for cholesterol(4) and phospholipid(5). Total lipid analyses according to the method of Bragdon(6) were also done upon the plasma samples of the rabbits infused with fat. Neutral fat was calculated by difference, merely by subtracting total cholesterol and total phospholipid from total lipid.

*Results.* 1. *Effect of sustained hyperphospholipidemia upon plasma cholesterol.* The immediate effect of the priming injection of 20 ml of phosphatide solution in the 3 control rabbits was an elevation of their average plasma phospholipid from 110 to 691 mg/100 ml as expected. No significant change was observed however in the plasma cholesterol concentration following this single injection. After 12 hours of continuous infusion of the same phosphatide solution into 6 rabbits, an increase of approximately 320% occurred in plasma cholesterol concentration. (Table I). Although the neutral fat content of plasma of these rabbits was not obtained, it was observed that no plasma sample obtained at end of infusion was lipemic. This suggests of course that no significant rise in neutral fat occurred(7). After 24 hours of continuous infusion, average plasma cholesterol of the 5 infused rabbits had increased approximately 120%. (Table I). The lesser rise in these rabbits as compared to that in the above rabbits was to be expected in view of a similar lesser elevation of the plasma phospholipid. Plasma samples obtained at end of infusion also were observed to be clear.

Injection and infusion of control rabbits receiving dextrose solution caused a slight rise in plasma phospholipid and no significant change in the plasma cholesterol level.

2. *Effect of sustained hypertriglyceridemia upon plasma cholesterol and phospholipid.* Immediately following the priming injection

‡ The apricot oil suspension was obtained from Abbott Laboratories. It consisted of 10% suspension of apricot oil containing 0.5% Tween and 5% dextrose.

TABLE II. Effect of Infusion of Emulsified Fat upon Plasma Cholesterol, Phospholipid and Triglyceride.

| | Rabbits infused with fat emulsion | Control rabbits infused with Tween (0.5%) and dextrose (5%) solution |
|---|---|---|
| No. | 8 | 3 |
| Avg wt (g) | 1793 (1496–2100) | 1782 (1603–1904) |
| Avg total amt fat given (g) | 5.2 (4.7–5.6) | — |
| Avg plasma cholesterol (100 mg/100 ml) | | |
| Before inj. | 62 ( 41– 96) ± 3.5* | 43 ( 34– 50) ± 3.2 |
| 10 hr after | 127 ( 82– 168) ± 13.8 | 49 ( 34– 56) ± 2.4 |
| Avg plasma phospholipid (100 mg/100 ml) | | |
| Before inj. | 120 ( 82– 168) ± 13.8 | 112 (104–120) ± 12.4 |
| 10 hr after | 199 (119– 341) ± 24.5 | 113 (104–122) ± 11.2 |
| Avg total lipid (100 mg/100 ml) | | |
| Before inj. | 308 (173– 576) ± 38.4 | 272 (250–296) ± 36.8 |
| 10 hr after | 1197 (552–2890) ±400 | 265 (245–300) ± 34.4 |
| Avg plasma triglyceride (mg/100 ml) | | |
| Before inj. | 100 ( 7– 162) ± 35 | 117 ( 94–142) ± 38 |
| 10 hr after | 842 (316–2332) ±250 | 111 ( 79–139) ± 32 |

* S.E. of mean. Fig. in parentheses give range of values.

of 20 ml of apricot oil into the 4 control rabbits, no change was observed in either plasma cholesterol or phospholipid concentration. Average plasma triglyceride concentration however rose from 80 to 513 mg/100 ml. After 10 hours of continuous infusion of the same fat emulsion into 8 rabbits, an increase of 105% occurred in the average plasma cholesterol concentration (Table II) and an increase of 66% in the phospholipid concentration. The 3 control rabbits injected and infused with the emulsifying medium alone exhibited no significant change in any plasma medium.

*Discussion.* The results obtained demonstrate that when the rabbit is injected and infused with either phosphatide or triglyceride in sufficient quantity to maintain a *continuous* state of hyperphospholipidemia or hypertriglyceridemia respectively, a hypercholesteremia quickly results. The rabbit thus resembles the rat(1) in the ease with which hypercholesteremia can be induced by this means.

The mechanism responsible for the hypercholesteremia induced by *sustained* infusion of either phosphatide or triglyceride remains to be elucidated. It appears probable however that the infusions do not lead to hypercholesteremia by interference with hepatic R-E cells by their possible colloidal contents. This is believed because hypercholesteremia does not occur with interference of R-E cell activity by particle administration unless large amounts of exogenously derived cholesterol are given at the same time(8). Then too, in the case of phosphatide injection at least, hypercholesteremia led to an excess of cholesterol deposited in the liver(3),—a phenomenon incompatible with the concept of possible severe interference with the function of the hepatic R-E cell.

*Summary.* Continuous elevation of plasma phospholipid in the rabbit by continuous infusion of a suitable phosphatide quickly leads to hypercholesteremia. A similar phenomenon was observed following elevation of plasma triglycerides by infusion of a fat emulsion. These results are in agreement with those observed in rats infused with similar substances.

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Received December 26, 1956. P.S.E.B.M., 1957, v94.

## Role of Vitamin $B_{12}$ in Nucleic Acid Metabolism. V. Liver Deoxyriboaldolase Activity.* (22976)

W. T. WONG AND B. S. SCHWEIGERT

*American Meat Institute Foundation and Department of Biochemistry, University of Chicago*

Vit. $B_{12}$ deficiency has been associated with decreased nucleic acid content in rat liver (1,2), and microorganisms(3,4). This decreased nucleic acid content may be related to defective carbohydrate metabolism in view of reported reduction in ability of the deficient animal to utilize carbohydrate(5). Liver contains an enzyme system for synthesis of ribose and deoxyribose by condensation of $C_2$ and $C_3$ compounds(6,7). Lowered rate of formation of $C_5$ sugars, and hence of nucleic acids in liver, of vit. $B_{12}$ deficient animals could, therefore, be due to lowered substrate concentrations of these $C_2$ and $C_3$ compounds, and/or to decreased activity of condensing enzymes to form the $C_5$ sugars. Accordingly, deoxyriboaldolase (DR-aldolase), the enzyme catalyzing the reaction: Glyceraldehyde-3-phosphate + acetaldehyde = Deoxyriboside-5-phosphate, was chosen for study.

*Materials and methods.* Vit. $B_{12}$ deficient rats of Sprague-Dawley strain were raised as previously described(2). Livers were removed from exsanguinated animals with or without previous ether anesthesia, and 1 g portions were immediately homogenized in 10 ml 0.2 M Tris buffer (pH 7.4) in Potter-Elvehjem glass homogenizer. The 10% homogenate was centrifuged at room temperature for 10 minutes, and the centrifugate used as enzyme source. Livers from deficient and supplemented paired littermates were used for each enzyme experiment. In initial experiments, each incubation tube contained: 0.2 ml 0.1 M Fructose-1, 6-diphosphate, 0.2 ml 0.06 M Sodium-iodoacetate, 0.1 ml 10% (v/v) Acetaldehyde, Enzyme preparation (0.2-1.4 ml), 0.2 M Tris buffer, pH 7.4, total volume of mixture = 2 ml. Fructose 1,6-diphosphate solutions were prepared fresh from Barium salt (Mann) by adding equivalent amounts of $Na_2SO_4$ and removing resulting $BaSO_4$ precipitate by centrifugation. After 30 minutes incubation at 37°C. with tubes stoppered, 2 ml 10% trichloracetic acid was added. The mixture was rapidly stirred, centrifuged, and aliquots of supernatant assayed for deoxyribose by the colorimetric method of Dische: 2 ml acetic acid-$H_2SO_4$ reagent containing 1% diphenylamine was added to 1 ml sample. Color was developed by heating mixture for 10 minutes at 100°C. Optical densities at 610 mu and 650 mu were determined in Beckman DU spectrophotometer, and the $\Delta$ O. D. (O. $D._{610}$ – O. $D._{650}$) compared to that of a standard DNA preparation. The findings are shown in Table I. The following modifications of above conditions for measuring enzyme activity were made in later experiments. An increased amount of sodium-iodoacetate (0.3 ml of solution not more than 6 weeks old) was used, and the solution of 10% acetaldehyde was made in 0.008M Tris buffer, pH 7.4. Better stoichiometry in the colorimetric assay was observed when 0.1 ml 10% acetaldehyde was added to every 30 ml acetic acid-$H_2SO_4$-diphenylamine reagent.

* Supported in part by National Live Stock and Meat Board and Division of Biology and Medicine, Atomic Energy Commission.

TABLE I. Relative DR-aldolase Activity of Livers from Vit. $B_{12}$-Deficient Rats.

| Age in wk | Sex | △ g body wt* | Relative activity† |
|---|---|---|---|
| 9 | ♀ | 39 | 71– 87 |
| 11 | ♀ | 9 | 74– 93 |
| 12 | ♀ | 45 | 55–100 |
| 17 | ♂ | 57 | 73– 74 |
| 21 | ♀ | 26 | 68– 75 |
| 22 | ♀ | 39 | 73– 85 |
| 23 | ♀ | 21 | 74–100 |

* Difference in body wt between each pair of supplemented and deficient littermates.

† In each case, activity of liver of supplemented rat was arbitrarily taken as 100%. The values denote the range observed with 2 or 3 enzyme concentrations; this range was reduced to 10% or less in subsequent experiments with use of modified procedure in enzyme assay (Table II).

Color developed at room temperature (30-36°C) for 15-20 hours according to the method of Burton(8). The dichromatic method of colorimetric assay was again employed, using deoxyribose† as the standard. Optical densities were measured at 600 mu instead of 610 mu, and at 650 mu. After these alterations in incubation and colorimetric procedures were made, it was possible to obtain better stoichiometry when the enzyme preparation was present in concentrations ranging from 0.2 to 1.0 ml.

*Results.* Less than 10% variation in results between each enzyme concentration was obtained within this range. Table II summarizes the results obtained with this modified procedure for another series of vit. $B_{12}$ deficient and supplemented animals. It can be concluded, from data presented above, that there is a definite decrease in enzymatic activity in livers of vit. $B_{12}$ deficient rats, as compared to that in livers of supplemented littermates. Earlier studies showed that the per cent protein in liver from vit. $B_{12}$ deficient rats is not altered(1). However, the nature of the crude enzyme system does not permit the conclusion that the DR-aldolase activity is indeed lower in the deficient animal. Since Fructose-1, 6-diphosphate is used as a source for triose-phosphate without exogenous aldolase added, the results may be interpreted as a change in either aldolase or DR-aldolase activity, or a combination of both. Further studies are needed to elucidate this point.

† California Foundation for Biochemical Research.

TABLE II. Apparent DR-aldolase Activity of Livers from Deficient (A1) and Supplemented (A3) Rats.

| Diet, A | Body wt, g | Age, wk | Sex | μg DR formed/g liver* | Relative activity |
|---|---|---|---|---|---|
| 1 | 204 | 36 | ♀ | 885 | 80 |
| 3 | 247 | | | 1110 | 100 |
| 1 | 230 | 36 | ♂ | 880 | 80 |
| 3 | 310 | | | 1197 | 100 |
| 1 | 250 | 37 | ♂ | 753 | 80 |
| 3 | 350 | | | 947 | 100 |
| 1 | 351 | 37 | ♂ | 803 | 73 |
| 3 | 459 | | | 1105 | 100 |
| 1 | 248 | 37 | ♀ | 662 | 68 |
| 3 | 284 | | | 977 | 100 |

* Avg of results from 3 enzyme concentrations.

*Summary.* An apparent decrease in liver deoxyriboaldolase activity was observed in livers from vit. $B_{12}$ deficient rats as compared to the activity observed with livers from littermates supplemented with vit. $B_{12}$.

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Received December 26, 1956. P.S.E.B.M., 1957, v94.

# Failure to Detect Estrogenic or Gonadotrophic Activity in Ergotoxine.* (22977)

M. C. Shelesnyak
*Department of Experimental Biology, Weizmann Institute of Science, Rehovoth, Israel.*

Ergotoxine produces manifestations of increased systemic estrogen in early pregnant and pseudopregnant rats(1). These include: termination of pregnancy and pseudopregnancy with the appearance of estrous type vaginal smears within 96 hrs; and the suppression of the decidual cell reaction(2). The injection of supplemental progesterone overrides or reverses the ergotoxine action(2,3). The ergotoxine effect may be due to direct estrogenic activity, indirect estrogenic by gonadotrophic (FSH) action, or by interference with progesterone utilization which would reflect itself as an increased estrogen status.

In attempting to elicit information on site and mechanism of the alkaloid drug action, tests for estrogenic and gonadotrophic activity were carried out in rats treated with ergotoxine. This is a report of the assays.

*Methods.* Assays for estrogenic activity were carried out on 40 spayed adult female rats (bd wt 180 ± 6.7 g), and 44 immature rats (22/23 days old; 47.0 ± 7.2 g). The immatures also serve for assay of gonadotrophic activity(4).

*Results.* 30 adult females were spayed and 5 days later injected with 0.5 mg ergotoxine methanesulphonate† (0.1 ml 95% ethanol); 10 females were primed with 1 γ stilbesterol 10 days after ovariectomy, and 5 days later injected with 0.5 mg ergotoxine twice daily x 3 days. Vaginal smears were examined daily during and for 7 days after treatment. No evidence of estrogenic activity was seen in smears, nor was there any observable change in the uterine status to indicate modification of hormonal deprivation.

44, 22/23-day-old immatures were divided into 28 ergotoxine, and 16 alcohol treated. Experimental animals received 1.5 mg ergotoxine in 0.2 ml 95% ethanol; controls received only alcohol. 72 hrs later uteri were removed, trimmed and weighed. Uteri of ergotoxine-treated rats weighed 66.5 ± 17.02 mg/100 g bd wt: control uteri were 66.1 ± 15.1 mg/100 g bd wt. There is no significant difference ($t = 0.08$; $P > 0.9$).

The weight response and histology of the ovaries of the immature rats (II) was used as an index of gonadotrophic activity. Ovaries of ergotoxine-treated rats weighed 47.8 ± 8.8 mg/100 g bd wt: and of control rats 50.6 ± 8.9 mg/100 g bd wt. There is no significant difference ($t = 1.010$; $0.4 > P > 0.3$). There is no evidence of gonadotrophic activity from ergotoxine. Microscopic study of H & E preparations revealed no structural differences between ovaries of ergotoxine-treated rats and controls.

*Conclusions.* Amounts of ergotoxine methanesulphonate which effectively terminate pregnancy, pseudopregnancy and deciduoma formation(1-3), failed to show estrogenic or gonadotrophic activity as tested in adult spayed, and immature intact rats. The termination of pregnancy and pseudopregnancy with a concomitant appearance of estrous configuration in the vaginal smears is therefore not due to any inherent estrogenic or gonadotrophic activity of the ergotoxine.

*Summary.* Ergotoxine methanesulphonate was tested on adult spayed, and immature intact rats, for estrogenic and gonadotrophic activity. None was found. The action of ergotoxine with respect to terminating early pregnancy and pseudopregnancy cannot be explained on the basis of estrogenic activity in ergotoxine.

It is a pleasure to express my gratitude to Mr. Shalom Meir Joseph for his technical assistance.

* Supported in part by grants from Population Council (N.Y.), and Sandoz, Basle.

† Ergotoxine methanesulphonate was an 1:1:1 mixture of ergocornine, ergocristine, and ergokryptine methane sulphonate, made available through the courtesy of Dr. A. Cerletti, Sandoz.

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Received December 27, 1956. P.S.E.B.M., 1957, v94.

## Comparison of Atrophy and Glycogen Storage in Some Muscles After Castration and Denervation.* (22978)

SAMUEL L. LEONARD
*Department of Zoology, Cornell University, Ithaca, N. Y.*

Castration in adult male rats causes atrophy and loss of glycogen in perineal muscles (including levator ani) and testosterone restores the muscles to normal(1,2). Denervation of skeletal muscle (gastrocnemius) also causes loss of glycogen, coincident with atrophy(3,4). The present study is concerned with a comparison of atrophic and glycogen changes which follow castration and denervation in the male hormone sensitive levator ani and cremaster muscles. The effect of some hormones on glycogen deposition in denervated muscles is also presented.

*Methods.* Adult male rats of matched body weights were employed, except where indicated, and all operations were performed using ether anesthesia. The genito-femoral nerve to the cremaster was evulsed just posterior to the ilio-lumbar vessels. The nerves forming the pudendal plexus on each side of animal were destroyed to denervate the perineal muscles. A section of the posterior division of the femoral nerve was removed to denervate the rectus femoris muscle. Wherever it was obviously feasible, the right muscle was denervated and the left one used as control, otherwise separate groups of rats were used. In experiments with hormone treatment, testosterone propionate (1 mg doses) and cortisone acetate (2 mg doses) were administered daily for 3 days prior to autopsy.† A 24-hour fast preceded the autopsies and muscles were removed from the anesthetized (nembutal) rats. Only the levator ani of the perineal muscle group was used because of its discrete nature. Varying periods of time elapsed after the operations and before autopsy. Weighing and glycogen determinations were made as previously described(5).

*Results.* The data in Table I show weight changes in the rectus femoris, cremaster and levator ani muscles following castration and denervation. Fifteen days after denervating the right rectus femoris, the muscle weighed 48% less than the control. Previous observations have shown no appreciable weight loss in this muscle after castration and no data are recorded.

The weight of the levator ani 15 days after either denervation or castration was less than the controls but denervation caused a greater loss in weight. In contrast, there was no significant weight loss in the cremaster 15 days after either denervation or castration.

On the assumption that a time factor might be involved in the latter instance, another group of rats was castrated and autopsied 32 days later. A significant weight loss in the cremaster now occurred. For the denervation experiment, young male rats (120-140 g body weight) were employed and 42 days elapsed after denervation of the right cremaster before autopsy. In addition, reference controls were obtained on day of operation by weighing cremaster muscles from a similar group of young males. The weights of the cremaster muscle 42 days after denervation were significantly less than the paired controls. How-

* Aided by grant from Muscular Dystrophy Assn. of America, and by Sage and Sackett Research Funds of Zoology Department, Cornell University.

† Hormones used in these experiments were generously given by Schering Corp., Bloomfield, N. J.

TABLE I. Comparison of Muscle Weights following Denervation and Castration.

| Exp. (No. of rats) | Period, days | Wt in mg ± S.E. Exp. | Control | % loss |
|---|---|---|---|---|
| | | (Rectus femoris) | | |
| Denervate ( 6) | 15 | 402 ± 23 | 769 ± 44 | 48 |
| | | (Cremaster) | | |
| Denervate ( 7) | 15 | 423 ± 27* | 486 ± 38 | 13 |
| " (10) | 42 | 313 ± 20† | 394 ± 14 | 21 |
| Castrate (10) | 15 | 509 ± 28* | 541 ± 21 | 6 |
| " (12) | 32 | 271 ± 15 | 426 ± 14 | 36 |
| | | (Levator ani) | | |
| Denervate (11) | 15 | 96 ± 6 | 197 ± 10 | 51 |
| Castrate (10) | 15 | 145 ± 12 | 211 ± 19 | 31 |

* Difference of means not significant; others $P < .02$.

† Avg initial wt of cremaster muscles (6 rats) at time of operation; right = 126 ± 8, left = 124 ± 8 mg.

ever, a comparison of weights of the reference control and the denervated muscles showed that the latter increased from an initial weight of 126 ± 8 mg to 313 ± 20 mg. Growth was greater in the contralateral muscle with intact nerves over the same period.

Although it has been shown that glycogen in the denervated muscle is always less than in the control whether rats are killed in a fed state or after a fast(4), the experiment was repeated using the rectus femoris and cremaster muscles. Six rats in each group were employed and the period of denervation was for 15 days. The percentage loss in glycogen from control levels in the rectus femoris in the fed and fasting state was 41% and 31% respectively. For the cremaster, it was 38% and 35% respectively. Although it appears to make little difference on a relative basis whether the animals are fed or starved, subsequent glycogen studies were made on the fasting animal.

After periods of 5, 15 and 42 days following denervation or 15 days after castration, the glycogen levels of the cremaster were all significantly lower than the controls (Table II). In the levator ani, castration and denervation also decreased glycogen concentration and, in both muscles, the effect of denervation was greater than castration at comparable periods.

The ability of testosterone and cortisone to

TABLE II. Muscle Glycogen Levels following Denervation and Castration.

| Exp. (No. of rats) | Period, days | Glycogen, mg % ± S.E. Exp.* | Control |
|---|---|---|---|
| | | (Cremaster) | |
| Denervate ( 6) | 5 | 359 ± 14 | 524 ± 10 |
| " ( 7) | 15 | 333 ± 11 | 476 ± 16 |
| " (10) | 42 | 365 ± 15 | 477 ± 27 |
| Castrate (10) | 15 | 423 ± 20 | 507 ± 13 |
| | | (Levator ani) | |
| Denervate (11) | 15 | 155 ± 9 | 387 ± 13 |
| Castrate (12) | 15 | 304 ± 17 | 396 ± 9 |

* In all experiments, difference of means significant, $P < .01$.

elevate glycogen in the rectus femoris and cremaster, denervated for 16 days, was studied. Because of their greater responsiveness to these steroids, the leg muscles of female rats were employed and cortisone was not used in castrated males since this hormone has no effect on the cremaster(6). Table III shows that both hormones increased glycogen levels in the denervated leg muscles but these steroids were 2-3 times more effective when the nerves were intact. Testosterone produced a substantial increase in the denervated cremaster of castrated males but not as great as in the intact muscle.

Since distention or stretch of the abdominal wall muscles of pregnant rats is sufficient to decrease their glycogen level(7), the possible effect of distention of the cremaster due to the physical presence of the testis was investigated. Ten rats were made unilaterally cryptorchid and 25 days later both cremaster muscles were assayed for glycogen. The glycogen of the muscle on the cryptorchid side

TABLE III. Hormone Effect on Glycogen Deposition in Denervated and Intact Muscles.

| Treatment (No. of rats) | Glycogen, mg % ± S.E. Denervated musc. Inj. | Control | Intact musc. Inj. | Control |
|---|---|---|---|---|
| | (Rectus femoris) | | | |
| T† (24) | 292±14 | 221± 6 | 542±15 | 380±17 |
| C (24) | 281±15* | 233±12 | 535±20 | 385±17 |
| | (Cremaster) | | | |
| T (24) | 459±27 | 322±11 | 648±33 | 450±11 |

* Difference of means, statistically $P = .02$; others $P < .001$.

† T = Testosterone; C = Cortisone.

was 505 ± 19 mg% and the control, 485 ± 21 mg %, showing no effect due to presence of testis.

*Discussion.* Although the cremaster and levator ani are male hormone sensitive muscles, the effect of denervation on weight loss is more pronounced in the latter. The data do not permit a similar comparison for castration effects but based on previous results, the relative weight loss about 1 month after castration is 61% for the levator ani(8) versus 36% for the cremaster. No difference in rate of denervation atrophy in 4 leg muscles of the rat is reported(9), but the laryngeal muscles regress faster than either the diaphragm or the external anal sphincter(10,11). It is not clear why there is a difference in rate of denervation atrophy among these skeletal muscles. In the levator ani, denervation causes a greater atrophy than castration within a comparable period of time but the experiments as designed, can only suggest that denervation is less effective than castration in the cremaster.

Growth of the cremaster continues after denervation in the young rat but at a rate slower than normal. Sections of 2 denervated muscles stained for nerve fibres gave no evidence of nerve regeneration by 42 days.‡ Part of the continued growth may be attributed to the male hormone. Some workers (12) state that testosterone increases the rate of denervation atrophy of the gastrocnemius in young males and others(13) report no significant effect of this hormone on the weight of the denervated anterior tibial muscle.

The decrease in glycogen concentration in the cremaster following denervation or castration precedes the loss in weight. In the levator ani, however, both the loss of glycogen and weight is observed to occur together. At comparable periods of time, the decrease in glycogen is greater after denervation than after castration in both muscles. The loss of glycogen after denervation occurs with great rapidity(4) and it is suggested that a reduced glycogen level offers an early criterion of the success of muscle denervation, *i.e.*, as in the cremaster.

‡ Dr. M. Singer kindly prepared these slides.

Testosterone and cortisone elevate glycogen in the denervated rectus femoris although the effect of cortisone is not great. The effect of testosterone on the denervated cremaster is very pronounced. Adrenal cortical extract did not alter the glycogen in denervated muscles of the adrenalectomized rat(14) but insulin elevated muscle glycogen provided glucose was given 3 hours after the hormone(4). Thus some hormones can stimulate glycogen deposition in denervated skeletal muscle; the effect of the nerve is quantitative, not conditioning.

*Summary.* Comparison of the weight and glycogen losses in the levator ani and cremaster muscles of the rat after castration and denervation show that (1) denervation atrophy is greater in the levator ani than in the cremaster, (2) denervation atrophy is greater than castration atrophy in the levator ani, (3) the loss of glycogen in both muscles after denervation is greater than after castration. Growth of the denervated cremaster continues in the young animal. Testosterone and cortisone can induce glycogen deposition in some denervated skeletal muscles; the effect of the nerves on this response is quantitative rather than conditioning.

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Received December 27, 1956. P.S.E.B.M., 1957, v94.

# Constitutional Nonhemolytic Hyperbilirubinemia in the Rat: Defect of Bilirubin Conjugation. (22979)

JOHN V. CARBONE AND GEROLD M. GRODSKY
(Introduced by Harold A. Harper)
*Department of Medicine, University of California School of Medicine, San Francisco.*

Gilbert(1) first described a condition in man in which the blood of an otherwise healthy individual shows a high content of indirect-reacting bilirubin. Since that time other authors have observed similar cases(2-4) and have concluded that a metabolic disturbance in the hepatic mechanism for clearing bilirubin from the blood is responsible(5,6). Several investigators have suggested that an analogous defect might also be responsible for certain frequently fatal forms of hyperbilirubinemia and kernicterus in newborn infants (7,8). A similar constitutional abnormality in bilirubin metabolism has also been observed in a mutant strain of the Wistar albino rat (9). It has been reported that bilirubin is excreted from the body primarily as conjugate of glucuronic acid(10,11). This is supported by recent studies on conjugation of bilirubin in liver and kidney homogenates in the rat(12) and in liver homogenates in man*, in which it was found that the conjugated pigment produced was destroyed by $\beta$-glucuronidase, contained one mole of glucuronic acid per mole of azo pigment, and could be assayed by the 1-minute "direct" bilirubin method(13). The following scheme has been proposed for the conjugation mechanism for glucuronides(14): glucose-1-phosphate + uridine triphosphate $\longrightarrow$ uridine diphosphate glucose $\xrightarrow{\text{DPN}}$ uridine diphosphate glucuronic acid (UDPGlu) + aglycone $\longrightarrow$ aglycone glucuronide. Lack of any enzymes or co-factors involved in this mechanism or impaired permeability of the cellular membrane to bilirubin could be responsible for the metabolic defect observed in constitutional nonhemolytic jaundice.

Tissue homogenates were utilized in the present study to investigate the enzymatic mechanisms involved in formation of glucuronides in liver and kidney of rats with constitutional nonhemolytic hyperbilirubinemia (CNH).

*Methods.* The incubation procedure, a modification of the method of Dutton and Storey(15), was similar to that previously described(12). A homogenate was prepared of 100 mg of liver or kidney tissue from a CNH rat/ml of isotonic potassium chloride containing $3.2 \times 10^{-4}$ M potassium bicarbonate. One ml of this suspension was added to buffer solution containing 0.3 ml of 0.5 M potassium phosphate (pH 7.4) and 0.1 ml of 0.3 M magnesium chloride. To this mixture was added 1 ml of boiled liver extract (15) as source of UDPGlu. Finally, either 40 $\mu$M of bilirubin suspended in 1 ml of 0.3% albumin solution (pH 7.4) or $1.4 \times 10^{-4}$ M *o*-aminophenol and $10^{-3}$ M ascorbic acid in 1 ml of the albumin solution were added to the incubation mixture. Incubations were maintained at 37° for 45 min. The amount of bilirubin conjugate produced was determined after centrifugation by measuring concentration of "direct" reacting azo pigment in the supernatant, using the method of Ducci and Watson(13). *o*-Aminophenol glucuronide was determined by the method of Dutton and Storey(15). Experiments with bilirubin and with *o*-aminophenol were made simultaneously with aliquots from the same homogenate sample. In each experiment a "zero-time" incubation served as control. Parallel experiments were made using tissue from normal rats of the Long-Evans strain of same weight and sex as CNH animals under investigation. All experiments were done in duplicate.

*Results.* The rats varied from 50 to 210 g in weight and from 4 to 15 weeks in age. The total serum bilirubin levels were 11 to 13 mg/100 ml and the 1-minute bilirubin levels varied from 0 to 0.3 mg/100 ml.

As shown in Table I, liver homogenates from CNH rats did not conjugate bilirubin.

* Unpublished observations.

TABLE I. Amounts of Conjugated Bilirubin and *o*-Aminophenol Produced by Homogenates of Tissue from Normal and CNH Rats (μg/100 mg Tissue).

| Material conjugated | Normal liver | | CNH liver | | Normal kidney with UDPGlu | CNH kidney with UDPGlu |
|---|---|---|---|---|---|---|
| | Without UDPGlu* | With UDPGlu* | Without UDPGlu | With UDPGlu | | |
| Bilirubin | | | | | | |
| 1 | 1.5 | 5.5 | .0 | .2 | 1.8 | .0 |
| 2 | 2.2 | 7.3 | .1 | .0 | 2.5 | .1 |
| 3 | 2.1 | 6.8 | .0 | .0 | | |
| *o*-Aminophenol | | | | | | |
| 1 | | 12.2 | | .0 | .0 | .0 |
| 2 | | 15.7 | | .0 | .1 | .0 |

* Refers to boiled liver extract as source of uridine diphosphate glucuronic acid.

Addition of boiled liver extract as a source of UDPGlu failed to stimulate conjugation in these tissue preparations, although it did cause a 3-fold increase in conjugative activity in homogenates of normal liver. In like manner, the conjugative activity observed in normal kidney tissue was lacking in kidney tissue from CNH rats.

Liver homogenates from CNH rats also failed to conjugate *o*-aminophenol (Table I). No conjugation of *o*-aminophenol was observed in kidney tissue from normal or CNH rats.

Attempts to inhibit endogenous β-glucuronidase by addition of $10^{-4}$ or $10^{-3}$ M concentrations of potassium saccharate failed to influence the conjugative activity in the abnormal tissues studied.

*Discussion.* Despite the presence of added bilirubin and UDPGlu, no conjugation was observed in a broken-cell preparation of liver from CNH rats. This evidence suggests that the defect in bilirubin metabolism in this condition is caused, at least in part, by impaired activity of glucuronyl transferase, the enzyme responsible for transfer of glucuronic acid from UDPGlu to the aglycone. This interpretation could be extended to include at lease the following two possibilities: 1) glucuronyl transferase activity may include several unknown enzymatic steps, any of which could be absent, 2) the defect may not be enzymatic, but may be caused instead by the presence of an inhibitor or absence of some co-factor. In addition, the demonstration of existence of one enzymatic defect does not necessarily infer that others further removed in the chain of glucuronide metabolism could not also be involved. A rapid breakdown of formed glucuronide by excess amounts of β-glucuronidase could be responsible for the observed results. Failure of potassium saccharate to influence conjugation, even when added in amounts up to 10 times that which normally inhibits β-glucuronidase(15), makes this possibility highly unlikely.

It is noteworthy that the metabolic defect is not specific to the liver, but is observed in the kidney of CNH rats as well. Conjugative activity for *o*-aminophenol is also absent in the liver of these animals. This observation suggests that an impairment of mechanism of glucuronide conjugation rather than a defect specific to metabolism of bilirubin is involved.

No comparison can be made between rate of conjugation of bilirubin and that of *o*-aminophenol since starting concentrations were different. Furthermore, any such comparison would be complicated by poor solubility of bilirubin and the toxic effects of *o*-aminophenol(15). The toxicity of *o*-aminophenol may explain why we and others(15) failed to find evidence of its conjugation in normal kidneys in spite of the presence of a small amount of conjugative activity in this tissue(16,17).

It is of interest to note that inability of CNH rats to conjugate and excrete bilirubin in a normal manner implies the existence of some as yet unknown alternative metabolic pathway for catabolism of hemoglobin or for removal of bilirubin.

The probable impairment of glucuronyl transferase activity in CNH rats is suggestive

evidence for the occurrence of this same defect in infants and adult man. The finding that a lack of glucuronyl transferase activity exists in the newborn mouse(18) suggests that a similar lack might be a factor in the frequent occurrence of nonhemolytic jaundice in human infants.

*Summary.* 1. Broken-cell preparations of liver and of kidney from rats with constitutional nonhemolytic hyperbilirubinemia failed to synthesize bilirubin or *o*-aminophenol glucuronides. 2. Inhibition of β-glucuronidase with potassium saccharate failed to influence conjugation of bilirubin in these animals. 3. The evidence presented suggests that in constitutional nonhemolytic hyperbilirubinemia in rats, glucuronyl transferase activity is absent or inhibited.

We are indebted to Dr. W. E. Castle, Department of Genetics, University of California, Berkeley, for supplying the CNH rats, to Dr. J. J. Eiler and Dr. B McIvor for generous permission to use their facilities.

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Received December 28, 1956. P.S.E.B.M., 1957, v94.

## Blood Pressure in Apparently Healthy Aged 65 to 106 Years. (22980)

ARTHUR M. MASTER, RICHARD P. LASSER AND HARRY L. JAFFE
*Department of Medicine, Mount Sinai Hospital, New York City*

A study of the range of the blood pressure in a large group of apparently healthy white Americans, 65 years of age and over, has just been completed. Little reliable information on this matter has hitherto been available. Previous studies were based on an inadequate number of subjects or covered an unrepresentative sample, of whom an unknown proportion suffered from cardiovascular disease. Little information also has been available on blood pressure in persons over 90 years of age. The study was conducted by means of questionnaires sent to physicians throughout the U.S. It was designed to insure that the population sample would 1) be large enough, 2) be widely distributed throughout the country, 3) cover a proper proportion of rural and urban dwellers, 4) be representative of various ethnic and economic groups, and 5) include only apparently healthy ambulatory people, living in the community, able to take complete care of themselves and without evidence of cardiovascular disease. The blood pressure data which have been obtained, therefore, characterize this select, active, healthy group. As such it is not a random population sample as would be obtained, for example, if every tenth person in the country had been studied.

Data gathered in such a random fashion would not, it is felt, answer the basic question of what range of blood pressure is comparable with, or is found in, active good health. The blood pressure in old people with heart disease and in institutions is being studied and the results will be published later. To minimize fluctuations in the blood pressure caused by emotional factors, the physicians were advised to take the patient's pressure after a short period of relaxation, to maintain a "matter-of-fact" attitude, and to record a newly taken pressure which had been carefully read to the nearest 2 mm Hg. In addition to the blood pressure reading, information was gathered on the geographic location of the community, and its size and nature, on the ethnic origin (place of birth) of the subject, on his height without shoes, his weight in the semi-nude, his occupation, employment or retirement, the degree of his physical activity and mental alertness, and the previous maximum blood pressure, if known. The geographic distribution of the subjects was comparable with that of all white persons, 65 years of age and over(1). There was a slight over-representation of subjects from the Northeastern States, but, since the mean values and standard deviations obtained from the 6 customary geographic areas of the United States(1) were similar, it was not considered necessary to "weight" the data in this regard. Of the 15,000 who had been examined, 5,757 (2998 males and 2759 females) were considered apparently healthy, as defined above. Analysis of the mean blood pressure and the standard deviation showed a high degree of homogeneity in the data from the various sources. These consisted of 1) individual practicing physicians, 2) members of the American College of Physicians, 3) members of the American Heart Assn., 4) physicians employed in Union Health Centers, 5) members of the American College of Chest Physicians, and 6) members of the Veterans' Administration. Since both the geographic and physician sources proved to be reasonably free from bias, this is the first study of an adequate number of apparently healthy white persons 65 years and older.

*Statistical analysis.* Arithmetic means, standard deviations, median and modal values were calculated for each sex, in each 5 year age group, from the 65th through the 94th year, and in the entire group 95 years of age and older. The statistical reliability of differences between mean values was determined from a computation of the standard error of the respective means. Frequency distribution graphs of the systolic and diastolic pressure of each sex in each 5 year age group were constructed. The tendency of physicians to record the blood pressure at the nearest zero, which was found in other large surveys(2a, 2b) was here, too, encountered. To minimize this artifact, class intervals of 10 mm Hg were adopted, with blood pressure readings ending in zero placed at the center of each interval. Thus, the systolic class intervals ranged from 85-94, 95-104, etc., up to 245-254. All values below 85 mm Hg systolic, and below 45 mm Hg diastolic, were each included in a single class interval. On the charts each class interval was entered at its zero midpoint, *e.g.,* 90, 100, etc.

*Results.* A. *Mean Pressure.* The mean systolic and diastolic pressures, and the standard deviations, calculated separately for each sex, in each 5 year age group, are shown in Table I. Unlike the trend at ages under 65, the mean blood pressures, both systolic and diastolic, *do not show a continuous rise* with age after 65. In men, the *average sys-*

TABLE I. Mean Blood Pressure of Apparently Healthy Aged People, 65 to 106 Years.

| Age group | No. cases | Systolic | Diastolic |
|---|---|---|---|
| | | Males | |
| 65- 69 | 911 | 143 ± 26.0* | 83 ± 9.9* |
| 70- 74 | 694 | 145 ± 26.3 | 82 ± 15.3 |
| 75- 79 | 534 | 146 ± 21.6 | 81 ± 12.9 |
| 80- 84 | 385 | 145 ± 25.6 | 82 ± 9.9 |
| 85- 89 | 325 | 145 ± 24.2 | 79 ± 14.9 |
| 90- 94 | 121 | 145 ± 23.4 | 78 ± 12.1 |
| 95-106 | 25 | 146 ± 27.5 | 78 ± 12.7 |
| | | Females | |
| 65- 69 | 856 | 154 ± 29.0 | 85 ± 13.8 |
| 70- 74 | 682 | 159 ± 25.8 | 85 ± 15.3 |
| 75- 79 | 464 | 158 ± 26.3 | 84 ± 13.1 |
| 80- 84 | 344 | 157 ± 28.0 | 83 ± 13.1 |
| 85- 89 | 263 | 154 ± 27.9 | 82 ± 17.3 |
| 90- 94 | 122 | 150 ± 23.6 | 79 ± 12.1 |
| 95-106 | 28 | 149 ± 23.5 | 81 ± 12.5 |

* ± stand. dev.

TABLE II. Values for Mean Pressures of All Apparently Healthy Aged People 65 to 106 Years.

| Males | Systolic | Coeff. of variation, % | Diastolic | Coeff. of variation, % |
|---|---|---|---|---|
| 2998 | 145 ± 22.3* | 15.4 | 82 ± 10 * | 12.2 |
| Females | | | | |
| 2759 | 156 ± 28.0 | 18.0 | 84 ± 14.7 | 17.5 |

* ± stand. dev.

*tolic* shows a small increase of 2-3 mm between ages 65-69 and the 70's, actually to 146 mm Hg, but thereafter is virtually stationary. In women the increase in systolic between 65-69 and 70-74, is somewhat larger—5 mm to 159 mm Hg, but thereafter the averages decrease steadily, and after age 90 are below the average for 65-69. The *average diastolic pressure* in both sexes shows little variation from ages 65 to 80, and tends to decline thereafter. The average attains a maximum in men at ages 65-69, *viz.* 83 mm Hg, and in women at ages 65-74, *viz.* 85 mm Hg. Though some variation in the *standard deviation* of systolic and diastolic pressure occurs among the various 5 year age groups, no systematic trend or age specific relationship exists. The mean systolic and diastolic pressures of each sex, as well as the standard deviation and coefficients of variation, have been determined for the entire sample population. These values are shown in Table II. The mean blood pressure of all the men was 145/82, of all the women, 156/84. In women, the systolic pressure is definitely higher and the diastolic pressure slightly higher than in men; the standard deviation and coefficient of variation are greater. The pulse pressure in women was 72 mm Hg and in men it was 63 mm Hg.

B. *Frequency Distribution Curves.* Frequency distribution curves of the systolic and diastolic pressure have been constructed for each sex, in 5 year age groups between 65 and 94, and one for those 95 and over (Fig. 1). All the curves have a basic bell-shaped pattern, which is associated with many biologic phenomena, although in common with some of them, most of the blood pressure curves are somewhat skewed to the right. This skewness is approximately the same for all

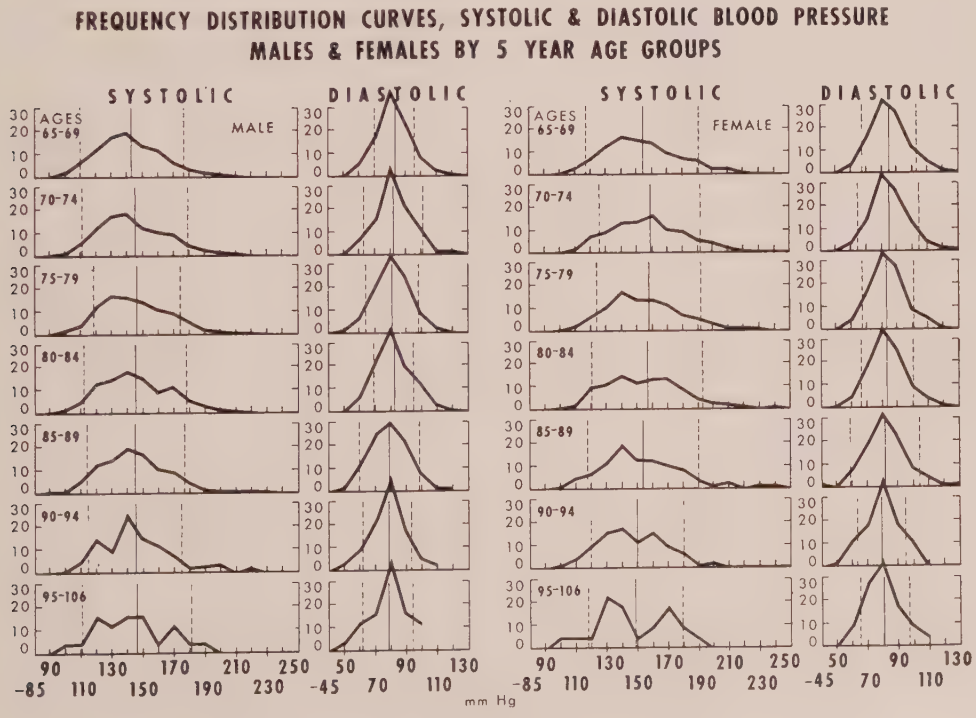


FIG. 1.

ages from 70 to 106 years of age, and thus differs from that found below the age of 65, and particularly between the ages of 45 and 64. During this age period the deviation of the blood pressure to the right progressively increases with age(3). The solid vertical line near the center of each curve represents the mean pressure; the 2 dotted vertical lines on either side of the mean, comprise in the area ± 1.282 sigma around the mean. This area includes approximately the middle 80% of all the values. In both sexes, all the *systolic pressure* curves have a positive skewness. In the males, they remain practically unchanged in basic contour at all ages, but in females, there are some minor changes. In females, also, there is a somewhat greater degree of skewness than in males at all ages over 65, and the middle 80% range is wider. The curves of the *diastolic pressure* of both sexes show no change in their basic characteristics with advancing age. In women, there is a somewhat greater dispersal of values from the mean.

C. *Range of Blood Pressure.* Two sets of limits have been calculated for systolic and diastolic pressure: the first includes all readings within ± 1.282 sigma of the mean, and the second includes all the readings within ± 2 sigma of the mean. The latter area encompasses approximately the middle 95% of all the values. Readings beyond the 2 sigma limit are outside the usual limits of variability and in our clinical experience are abnormal. Calculations of these 2 limits were made separately for each sex in each age group. There were no significant changes with age in the diastolic pressure range in either sex, or in the systolic pressure range in males. The female systolic limits showed variations of minor degree. Accordingly, in proposing practical standards of blood pressure for general use, a single set of standards for each sex is applicable to all ages, 65 and over, and these have been computed from the data and are shown in Table III. The blood pressure values have been adjusted very slightly to the figures customarily used by physicians in recording blood pressures. On this basis the middle 80% range for males is $\frac{115\text{-}175}{70\text{-}95}$; for females it is $\frac{120\text{-}192}{65\text{-}102}$. The middle 95% range for males is $\frac{100\text{-}190}{62\text{-}102}$; for females it is $\frac{100\text{-}212}{55\text{-}112}$.

TABLE III. Range of Blood Pressure in Apparently Healthy, Aged 65 to 106 Years.

| | Systolic (mm Hg) | | Diastolic (mm Hg) | |
|---|---|---|---|---|
| | ♂ | ♀ | ♂ | ♀ |
| Middle 80% (± 1.282 sigma) | 115-175 | 120-192 | 70- 95 | 65-102 |
| Middle 95% (± 2 sigma) | 100-190 | 100-212 | 62-102 | 55-112 |

Values have been very slightly adjusted to conform with readings customarily used by physicians.

*Discussion.* The peak average systolic blood pressure is reached before age 75 and the peak average diastolic before age 70. The reason for the absence of further increase in old age can only be speculated upon. It is probable that persons with high blood pressure, as they grow old, develop clinical cardiac disease at an increasingly rapid rate and a decreasing proportion of them, therefore, are found among the apparently healthy without heart disease, which alone are included in the present study. The selective factors in morbidity and mortality tend to maintain a constant or declining pressure in healthy old age.

The higher systolic pressure found in women in this study has been reported previously(4). This sex difference is first observed at about the age of 45, and is more or less coincident with the average age of onset of menopause. However, the fact that healthy women have higher pressures on the average than men, and yet have a longer life expectancy, indicates that a single set of blood pressure standards cannot be used for both sexes.

*Summary.* 1) A study has been made of the blood pressure in 2,998 males and 2,759 females, 65 to 106 years of age, who were apparently healthy and without known heart disease. 2. The mean blood pressure was 145/82 for men and 156/84 for women. 3. The mean systolic and diastolic blood pressures do not show a continuous rise with age after 65. In men the *peak systolic* is 145-146 mm Hg, in women, 159 mm Hg. After the

age of 74, the systolic pressure declines slowly in women, but remains essentially constant in men. 4. The mean *diastolic pressure* shows little variation from ages 65 to 80, and tends to decline thereafter. 5. *Frequency distribution curves* of systolic and diastolic pressures, at all ages, have the basic pattern of a bell-shaped curve. In both sexes, the curves have a positive skewness larger and more consistent in the systolic than in the diastolic pressures. 6. A single set of blood pressure standards has been computed for each sex, applicable to the entire apparently healthy population from age 65 to over 100. These computations place the middle 80% range ($\pm$ 1.282 sigma) in males at $\frac{115\text{-}175}{70\text{-}95}$, and females $\frac{120\text{-}192}{65\text{-}102}$; and the middle 95% range ($\pm$ 2 sigma) in males is $\frac{100\text{-}190}{62\text{-}102}$, in females $\frac{100\text{-}212}{55\text{-}112}$.

We gratefully acknowledge the help given us by Herbert H. Marks, Manager of Insurance Medical Statistics, and by Edward Lew, Actuary and Statistician of the Metropolitan Life Insurance Company, New York City.

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Received January 3, 1957. P.S.E.B.M., 1957, v94.

## Turnover and Nature of Fecal Bile Acids in Germfree and Infected Rats Fed Cholic Acid-24-$^{14}$C. Bile Acids and Steroids 41.*† (22981)

B. E. GUSTAFSSON, S. BERGSTRÖM, S. LINDSTEDT AND A. NORMAN
(Introduced by O. Wintersteiner)
*Departments of Histology and Physiological Chemistry, University of Lund, Sweden.*

The turnover of bile acids has been studied in normal rats(2) and in rats treated with chemotherapeutics(3). It was found that the rate of excretion was much slower in rats treated with chemotherapeutics than in normal animals. The purpose of the present investigation was to study excretion of cholic acid in rats reared in germ-free conditions from birth and in rats with a known intestinal flora. Furthermore, the nature of the fecal acids excreted after feeding 24-$^{14}$C-cholic acid was to be studied as a complement to earlier experiments carried out on normal rats(4), on rats treated with chemotherapeutics(5) and *in vitro* with microorganism isolated from rat feces(6).

*Methods.* The germfree rats used had been delivered into the germfree unit through cesarian section and hand-fed for the first 20 days according to the technic of Gustafsson (7) with slight modifications. Litter mates were reared outside the units on the same sterilized diet. The animals used were 3-6 months old. 3 germfree and 3 control animals were studied. The diet contained casein 22%, wheat starch 63%, arachis oil 10%, salt mixture 4% and sufficient amounts of vitamins. The diet was mixed with 50% water and autoclaved at 121°C for 20 minutes. 1-2 mg of sodium salt of cholic acid-24-$^{14}$C(8) (3.9 $\mu$c/mg) was autoclaved in water solution, transferred to the apparatus and given by mouth to the animals. The rats were kept in metabolic cages and their feces collected every 24 hours. The

* A preliminary report was read at meeting of Norwegian Biochem. Soc. in Oslo, June 1956(1).

† This work has been supported by Statens Medicinska Forskningsråd and Knut och Alice Wallenbergs Stiftelse, Stockholm.

feces were crushed and boiled twice in 80% ethanol for 3 hours. The isotope content was determined on a small aliquot of the combined extracts as described earlier(3). Prior to the chromatographic separations the ethanol extracts were evaporated *in vacuo* and extracted with butanol from an acidified water solution. The butanol extracts were subjected to reversed phase partition chromatography on hydrophobic Supercel(9,10). Phase system C: Stationary phase: isooctanol/chloroform 1:1; Moving phase: methanol/water 1:1. 4 ml of the stationary phase was used per 4.5 g of supporting medium. Fecal excretion of cholic acid in germfree rats during a 10 day period was determined. The combined ethanol extracts were saponified and cholic acid isolated with column chromatography and then quantitatively determined by the method of Sjövall(11).

*Results.* 1. *Nature of fecal bile acids after feeding 24-$^{14}$C-cholic acid.* When labelled cholic acid was fed the acids appearing in the feces of rats in normal environments were found to consist of a variety of compounds (4), while in rats treated with chemotherapeutics(5) only taurocholic acid was found, *i.e.* the same compound that was found in the bile when labelled cholic acid had been injected(12). The changes brought about by the intestinal microorganisms were a splitting of the peptide bond of taurocholic acid and alterations on the steroid nucleus. Some strains of microorganisms capable of splitting

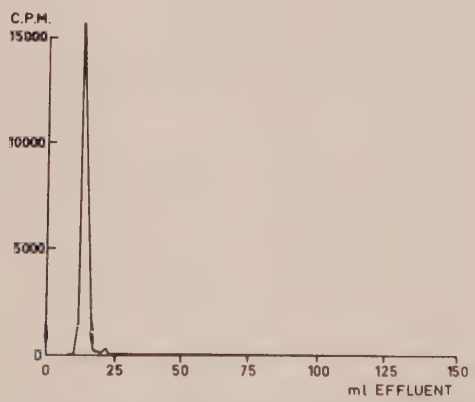


FIG. 1. Chromatogram of labelled compounds in feces of rat 82 × 1 under germfree conditions. Phase system C.

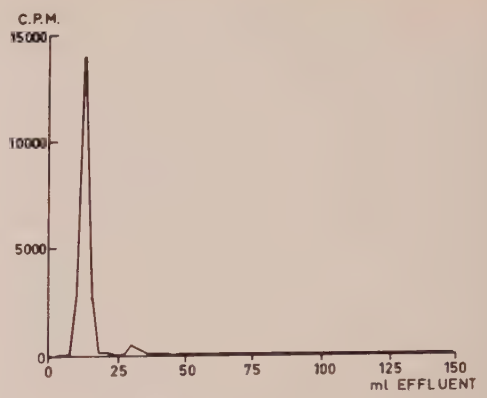


FIG. 2. Chromatogram of labelled compounds in feces of rat 82 × 1 infected with *Aspergillus niger*. Phase system C.

the peptide bond *in vitro* have been isolated (6), while the nature of the microorganisms effecting the attack on the steroid molecule is still largely unknown.

In Fig. 1 a chromatogram with phase system C of the fecal acids from a germfree rat (82 x 1) fed labelled cholic acid is shown. The activity is confined within one single peak appearing at the place of taurocholic acid. The material constituting this peak has been collected, hydrolyzed and re-run with carrier cholic acid in phase system C, where all of the activity appeared at the place of cholic acid. The results are identical with those earlier obtained in rats treated with chemotherapeutics(5), thus definitely showing that intestinal enzymes do not hydrolyze the conjugated bile acids. Identical results were obtained with the 2 other germfree animals studied.

One of the germfree rats (82 x 1) was accidentally infected with *Aspergillus niger*. 15 days later a dose of labelled cholic acid was given. Fig. 2 shows a chromatogram of the fecal acids of this rat. It is identical with that obtained from the same rat during germfree conditions, demonstrating the inability of the mold to attack either the peptide bond or the steroid part of the molecule. To study the effect of a strain of bacteria known to produce enzymes capable of hydrolyzing the peptide bond *in vitro*(6) we infected the same rat with *Clostridium perfringens type E.* After infection the animal had a slight diar-

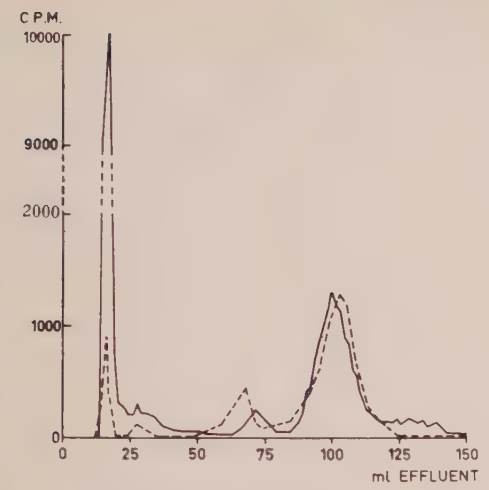


FIG. 3. Chromatograms of labelled compounds in feces of rat 82 × 1 infected with *Aspergillus niger* + *Clostridium perfringens* type E. Dotted line: Result of *in vitro* experiment with the same microorganism (redrawn from Norman and Grubb (5)). Phase system C.

rhea for a few days. The feces contained a large number of living microorganisms of the two strains in question. Fifteen days after the infection with *Clostridium perfringens* labelled cholic acid was administered as earlier. The chromatogram of the fecal acids is shown in Fig. 3 together with the result of an *in vitro* experiment with the same strain of bacteria. The peak that is seen at 80-110 ml effluent appears at the place of free cholic acid, while a small peak just before (50-75 ml) is caused by an unknown metabolite.

The rat (82 x 1) was finally removed from the unit and placed in the laboratory for 10 days to acquire a "normal" intestinal flora. The result (Fig. 4) is similar to that found in normal animals(4) with extensive splitting of the conjugates and further modifications of the free bile acids.

2. *Turnover of bile acids in germfree rats.* Elimination of cholic acid in rats treated with chemotherapeutics is much slower than in control animals(3). Rats treated with chemotherapeutics might not represent a physiologically steady state owing to the fairly short time of treatment and the possible toxic effect of the drugs(3). In the present work, however, no such objection can be raised and rate of elimination should therefore be considered equal to rate of synthesis. Fig. 5 shows a plot of $-\log\left(1-\frac{U_t}{U_{max}}\right)$ *vs* time for germfree and control animals. ($U_t$ = amount of activity excreted at time t. $U_{max}$ = plateau level reached in control animals—85-100% of injected amount—and total amount administered to germfree rats, where no plateau level was reached in the experimental period.) For a discussion of this way of plotting the result see ref. 2. The half-lives that can be read from this figure/normal: 2 days (1.2-2.1); germfree: 11.4 (8-14.5)/ show that the half-lives of germfree rats are of the same

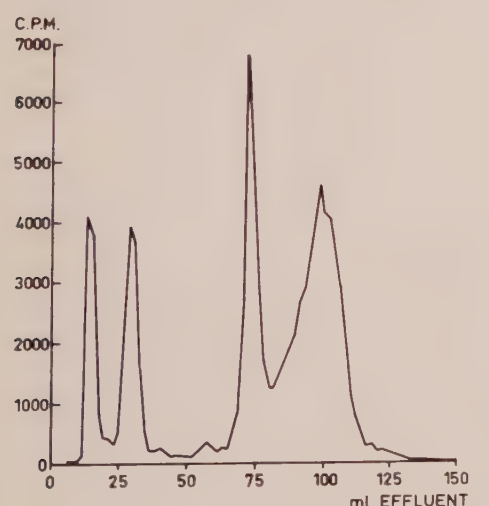


FIG. 4. Chromatograms of labelled compounds in feces of rat 82 × 1 in normal environments. Phase system C.

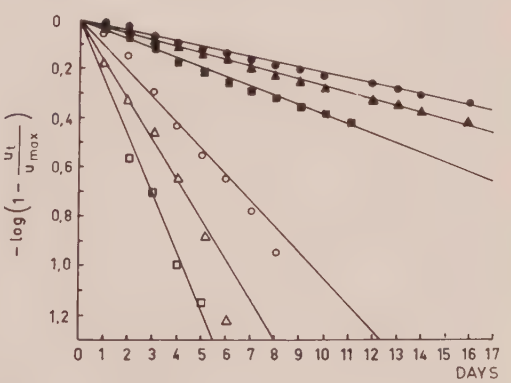


FIG. 5. Semilogarithmic plot of elimination of cholic acid in normal (□ △ ○) and germfree (■ ▲ ●) rats.

order of magnitude as in rats treated with chemotherapeutics.

To obtain the daily synthesis of bile acids one would have to know the size of the bile acid pool of the germfree rats. It has not been possible to determine this by direct methods as done in normal rats(13). However, as taurocholic acid is excreted unchanged in germfree rats it was possible to determine the daily excretion with the quantitative paper chromatographic method of Sjövall(11). This was done for a 10 day period in each rat. The mean daily excretion of cholic was 0.9 mg/100 g body weight corresponding to a pool of 15.4 mg/100 g body weight. While the size of the pool is considerably larger than found in normal animals by Bergström and Eriksson(13) daily excretion of cholic acid is less than in normal animals(2).

Germfree animals have been found to have a greatly distended coecum(7). The possibility that the bile acids are trapped in the coecum was excluded by an experiment where the distribution of activity in different parts of the intestinal tract was determined 24 hours after administration of labelled cholic acid. The result (Table I) shows that more than 80% of the recovered activity is located in the small intestine. This distribution is similar to that found in normal animals.

It was further shown by X-ray studies that a barium meal reached the colon in approximately the same time in a germfree and a control animal. Thus after 4 hours 2 fecal pellets were observed in both the control and the germfree animal.

3. *Influence of different infections on rate of turnover of bile acids.* Fig. 6 shows a semilogarithmic plot of excretion of isotope in rat 82 x 1 during periods with different conditions in the intestine. When the bile acids of this

TABLE I. Distribution of Isotope 24 Hours after Oral Administration of Cholic Acid-24-$^{14}$C to a Germfree Rat.

| Part of intestinal tract | % of isotope recovered |
|---|---|
| Duodenum | 3.5 |
| Small intestine | 83 |
| Coecum | 11.2 |
| Colon | 2.3 |

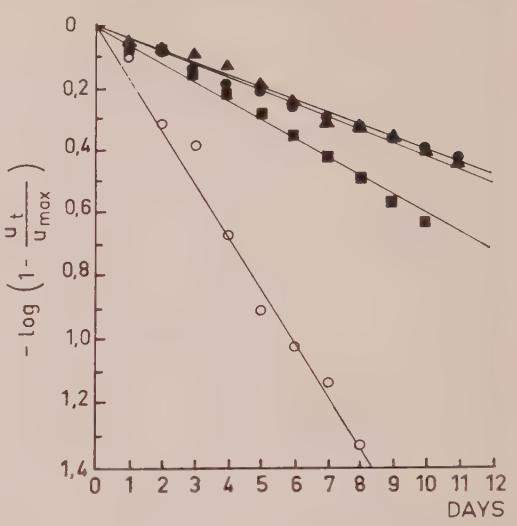


FIG. 6. Semilogarithmic plot of elimination of cholic acid in rat 82 × 1 during different conditions (●—● germfree, ■—■ infected with *Aspergillus niger*, ▲—▲ infected with *Aspergillus niger* + *Clostridium perfringens*, ○—○ totally infected).

rat in germfree surroundings had a half-life of 7 days monoinfection with *Aspergillus niger* did not produce any certain change in the half-life (5.2 days) or in fecal bile acid composition, nor did a superimposed infection of the same individual rat with *Clostridium* change the half-life of the cholic acid (8 days) in spite of a considerable splitting of the conjugates. When the infected rat finally was brought outside the apparatus and had acquired a "normal" intestinal flora the half-life of the cholic acid was reduced to that of a normal rat, *i.e.* 1.8 days.

*Summary.* 1. Taurocholic acid-24-$^{14}$C was the only metabolite found in feces of germfree rats fed cholic acid-24-$^{14}$C. 2. The half-life of cholic acid in germfree rats is 11.4 days as compared with 2 days in control animals. Daily excretion of cholic acid in germfree rats is 0.9 mg/100 g body weight. 3. Monoinfection of a germfree rat with *Aspergillus niger* did not change the half-life nor the composition of the bile salts in feces. Infection of the same rat with *Clostridium perfringens* resulted in free cholic acid in feces but no change in turnover time. When the rat was taken out of the germfree rearing ap-

paratus turnover time and bile acid picture changed to that of "normal" rats.

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Received January 3, 1957. P.S.E.B.M., v94, 1957.

## Antral Inhibition of Gastric Secretion.* (22982)

PAUL H. JORDAN, JR., AND BERNARD F. SAND
(Introduced by Edward R. Woodward)
*Departments of Surgery, Wadsworth General Hospital, Veterans Administration Center, Los Angeles, Calif. and University of California Medical Center, Los Angeles.*

Edkins(1) proposed that presence of secretagogues in the stomach promoted gastric secretion by release of a hormone, "gastrin," from the antral mucosa. Although his experiments were inadequate to prove this hypothesis, subsequent studies have demonstrated that the gastric phase of gastric secretion is mediated by a hormonal mechanism which is initiated by chemical(2) and mechanical(3) stimulation of the antral mucosa. The presence of acid of sufficiently strong concentration within the antral portion of stomach will diminish the rate of gastric secretion which results when antral gastrin mechanism is stimulated by secretagogues within the antrum(4). Whether the acid environment is unsuitable for release of gastrin or whether it results in production of a substance which actively inhibits production of HCl is not clearly established. Woodward (4), interested in the possible existence of a gastric secretory inhibitor factor from the antrum, was unable to inhibit the secretory effect of histamine by perfusion of the isolated, innervated antrum with acid and concluded that such a factor did not exist. Recently Harrison *et al.*(5) utilizing the technic of a divided antrum have presented evidence for existence of a gastric secretory inhibitor mechanism within the antrum. We have attempted to prove the existence of this mechanism by using the divided antral pouch technic in such a way that the environment of each antral segment may be altered simultaneously at will.

*Method and procedure.* Four healthy adult mongrel dogs were prepared as follows: The gastric antrum was removed from the gastrointestinal continuity and divided longitudinally forming 2 small antral pouches which were marsupialized. A Heidenhain pouch was constructed and drained by a

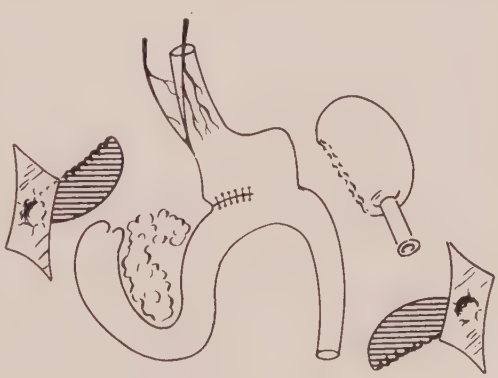

FIG. 1. Experimental preparation of stomach consisted of a Heidenhain pouch, 2 marsupialized antral pouches and a gastrojejunostomy.

* Supported by Grant, U.S.P.H.S.

stainless steel cannula for collection of gastric juice. Gastrointestinal continuity was restored by gastrojejunostomy. A wedge of the stomach between antrum and fundus was discarded to insure against the presence of antral mucosa in the Heidenhain pouch or acid secreting cells within the antral pouches. The final preparation is represented by Fig. 1. After recovery periods of 3 weeks, the animals were subjected to the following study. After a 12 to 16 hour fast, a dog was placed in a Pavlov frame and gastric juice was collected from the Heidenhain pouch every 30 minutes. Following 2 control periods, perfusion of 10% alcohol, used as stimulant for gastric juice secretion(6) was begun, in either of the antral pouches and was continued in the same pouch throughout remainder of experiment. After a sustained secretory response was obtained with alcohol, perfusion of 0.1N HCl was begun in the other antral pouch. Perfusion of alcohol in the first pouch and acid in the second pouch was continued until the HCl response from the Heidenhain pouch was completely or significantly inhibited. In all but 2 experiments which demonstrated inhibition of gastric secretion, physiological saline was substituted for perfusion of acid as the third phase of the study. Perfusions of alcohol and saline were continued to see if the secretory response to alcohol would return. The free HCl in gastric juice was titrated with 0.1N NaOH with Toeppfer's reagent as indicator. The amount of free HCl for each collection period was expressed in milliequivalents (mEq).

*Results.* Twenty-nine experiments were performed on 4 dogs. Alcohol failed to initiate gastric secretory response in 6 studies, (5 of these occurred in 1 dog). These experiments were terminated and are not considered in this analysis. In 17 of the remaining 23 studies, secretory response to perfusion of alcohol in one antral pouch was inhibited by perfusion of HCl in the second antral pouch. A perfusion of saline was substituted for perfusion of acid in 15 of the 17 experiments exhibiting gastric secretory inhibition. A return of free acid or an increase in acid secretion occurred in 10 of these studies. The 5 experiments that failed to demonstrate a significant return of gastric secretion occurred in 1 dog.

TABLE I. Mean HCl Production in mEq for Last 2 Periods of Each Experimental Phase.

| Dog | | I Alcohol | II Alcohol-acid | III Alcohol-saline |
|---|---|---|---|---|
| I | 1 | .15 | .08 | .63 |
| | 2 | .40 | .10 | .25 |
| II | 1 | 1.73 | .14 | .85 |
| | 4 | .10 | .02 | .09 |
| | 6 | .69 | .02 | .10 |
| III | 1 | .42 | .05 | .37 |
| | 2 | .85 | .02 | .10 |
| | 3 | 1.12 | .08 | .44 |
| | 4 | .13 | .01 | .20 |
| IV | 1 | 1.69 | .24 | 0 |
| | 2 | .07 | 0 | .26 |
| | 4 | 1.40 | .03 | 0 |
| | 5 | .15 | .02 | .01 |
| | 7 | .38 | .03 | 0 |
| | 8 | 1.48 | .02 | .01 |

Five of the 23 experiments, demonstrating an initial secretory response to alcohol, failed to display gastric secretory inhibition when perfusion of the second antral pouch with hydrochloric acid was performed.

The magnitude of the response to alcohol stimulation or acid inhibition was not related to the antral pouch employed for these perfusions except when there was a marked discrepancy in size. Then, the magnitude of alcohol secretory response and the acid inhibitory response depended upon whether the large pouch was employed for alcohol or acid perfusion respectively.

To analyze the results statistically, the average HCl output for the last 2 periods of each of the 3 phases of an experiment was considered the maximum secretory or inhibitory response obtained for the respective

TABLE II. Analysis of Variance between Phases I, II, and III.

| Source of variation | Degrees of freedom | Sum of squares | Mean square | F | P |
|---|---|---|---|---|---|
| Between phases of exp. | 2 | 3.59 | 1.79 | 20.8 | <.01 |
| Between exp. | 14 | 2.50 | .18 | | |
| Discrepance | 28 | 3.77 | .086 | | |
| Total | 44 | 9.86 | | | |

TABLE III. Mean HCl Production in mEq for Last 2 Periods of Experimental Phases I and II.

| Dog | | I Alcohol | II Alcohol-acid |
|---|---|---|---|
| I | 1 | .15 | .08 |
| | 2 | .40 | .10 |
| | 3 | .64 | .02 |
| II | 1 | 1.73 | .14 |
| | 2 | .10 | .09 |
| | 3 | .42 | .60 |
| | 4 | .10 | .02 |
| | 5 | .25 | .50 |
| | 6 | .69 | .02 |
| III | 1 | .42 | .05 |
| | 2 | .85 | .02 |
| | 3 | 1.12 | .08 |
| | 4 | .93 | .61 |
| | 5 | .13 | .01 |
| | 6 | .42 | .57 |
| IV | 1 | 1.69 | .24 |
| | 2 | .07 | 0 |
| | 3 | 1.10 | 1.13 |
| | 4 | 1.40 | .03 |
| | 5 | .15 | .02 |
| | 6 | .44 | .09 |
| | 7 | .38 | .03 |
| | 8 | 1.48 | .02 |

phases. Thus expressed, the data for the 15 experiments in which all 3 phases of study were performed, are seen in Table I. In Table II the analysis of variance of these data indicates that there is a significant difference in HCl production between the 3 phases of the experiment ($P<.01$). In a similar manner, the data for phases 1 and 2 of all 23 experiments are tabulated in Table III and the analysis of variance of these data (Table IV) indicates that there is a significant difference in gastric secretion between these two phases of the experiment ($P<.01$).

*Discussion.* Evidence presented by Harrison *et al.*(5) suggests that the antrum may actively inhibit production of gastric HCl which is mediated by chemical stimulation of the antral gastrin mechanism. The evidence presented by these authors suggests the hypothesis that reduction of pH within the antrum may actively inhibit gastric secretion by liberation of an inhibitory substance. The results of the present study using the double antral pouch preparation described above, provide direct evidence to support this hypothesis. In 17 of 23 experiments reported, the antral gastrin mechanism initiated by perfusion of one antral pouch with alcohol was inhibited by perfusion of the second antral pouch with HCl.

TABLE IV. Analysis of Variance between Phases I and II of All Experiments.

| Source of variation | Degrees of freedom | Sum of squares | Mean square | F | P |
|---|---|---|---|---|---|
| Between phases of exp. | 1 | 2.44 | 2.44 | 15.25 | <.01 |
| Between exp. | 22 | 4.53 | .206 | | |
| Discrepance | 22 | 3.57 | .16 | | |
| Total | 45 | 10.53 | | | |

The time required to inhibit the antral gastrin mechanism with antral perfusion of HCl varied from 1 to 3 hours. The reason for the wide variation in time to initiate the inhibitory mechanism is not clear. It has been our opinion that strength of the antral gastrin mechanism and the antral inhibitor mechanism may be dependent upon size of antral pouch perfused with alcohol and acid respectively.

The demonstration that gastric secretion is inhibited by antral perfusion with HCl can best be interpreted as the result of a humoral mechanism. It is our opinion that this mechanism is of a hormonal nature; however, further studies are required to elucidate this question. The importance of the gastric secretory inhibitor factor in the control of HCl production in normal and pathological states must be evaluated.

*Summary.* A method has been devised for making a Heidenhain pouch and 2 separate, marsupialized, antral pouches in the dog. Studies performed on 4 dogs prepared in this way indicate that reduction of pH within one antral pouch will inhibit production of HCl in the Heidenhain pouch when the latter is under stimulation by perfusion of alcohol in the second antral pouch. It is assumed that gastric inhibition produced in this manner demonstrates release of an inhibitory hormone by the antrum; however, this awaits further study for clarification.

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Received January 10, 1957. P.S.E.B.M., v94, 1957.

## Different Fractions of Plasma Proteins in Some Infectious Diseases. (22983)

SACHCHIDANANDA BANERJEE AND KANTI PADA CHATTERJEE
*Department of Physiology, Presidency College, Calcutta, India.*

Changes in plasma protein patterns usually manifest themselves by increase or decrease in concentrations of normal components or by appearance of protein fractions not seen under normal conditions. Different fractions of plasma proteins have been determined in different diseased conditions(1-6) but such studies in patients suffering from acute infectious diseases like cholera, small pox, tetanus and meningitis have not been published. The present communication deals with such studies in patients suffering from cholera, tetanus, small pox and meningitis by paper electrophoresis. Similar studies were also carried out in normal Indians for comparison.

*Methods.* Blood samples were collected in vials, containing crystals of potassium oxalate, from the anticubital vein as soon as patients suffering from small pox, tetanus and meningitis were admitted to the hospital. In patients suffering from cholera, blood samples were withdrawn when dehydration of the body was controlled by transfusion of saline and patients had normal urination. Normal subjects were students of our department of physiology. Total nitrogen in .1 cc plasma and nonprotein N in tungstic acid blood filtrate equivalent to 0.5 cc plasma were determined by the micro-Kjeldahl method. By multiplying the difference between the total N and nonprotein N with 6.25, total protein value of blood plasma was obtained. Fibrinogen was determined in 1 cc plasma by isolation as fibrin according to the method of Cullen and Van Slyke(7) and determination of N in fibrin by the micro-Kjeldahl method. 1 cc plasma was diluted 3 times with barbiturate-barbituric acid buffer of pH 8.6 and ionic concentration .05. .02 cc of the dilute plasma was applied to paper strip and electrophoretic separation was carried out for 12 hours by using L.K.B. paper electrophoresis apparatus with current of 6 mA and 300 volts. The filter paper strips were dried at 100°C, immersed in .1% bromophenol blue in absolute ethanol saturated with mercuric chloride for 30 minutes, washed in .5% acetic acid, dried, optical density curves of the different colored zones plotted using Photovolt Photoelectric Densitometer Model 425 on graph paper, and areas of component sections of graph were measured by cutting out the curves and weighing in micro balance. Total area of plotted curve was equivalent to total plasma protein content of sample used for electrophoresis, determined by the micro-Kjeldahl method. Weight of component sections of curves was proportional to area and protein value of each component was calculated from total protein value. As $\gamma$-globulin and fibrinogen zones in the electrophorogram were almost inseparable, the former was determined by deducting the sum total of albumin, $\alpha$-globulin, $\beta$-globulin and fibrinogen values from the total protein value. The results are given in Table I.

TABLE I. Different Fractions of Plasma Proteins (%).

| Subjects | Total protein | Albumin | α-globulin | β-globulin | γ-globulin | Fibrinogen |
|---|---|---|---|---|---|---|
| Normal (22) | 8.22 ± .1* | 4.64 ± .11 | 1.11 ± .06 | .77 ± .05 | 1.39 ± .05 | .31 ± .02 |
| Cholera (16) | 6.39 ± .10 | 2.26 ± .08 | .84 ± .05 | 1.37 ± .08 | 1.47 ± .09 | .45 ± .02 |
| | | | | ($B_1$) .79 ± .07 | | |
| | | | | ($B_2$) .71 ± .04 | | |
| *t* | 12 | 17.1 | 3.5 | 6.3 | .8† | 6.1 |
| Small pox (10) | 6.33 ± .06 | 2.40 ± .14 | 1.02 ± .08 | .89 ± .08 | 1.78 ± .12 | .44 ± .02 |
| | | | | | ($\gamma_1$) 1.13 ± .11 | |
| | | | | | ($\gamma_2$) .65 ± .05 | |
| *t* | 5.9 | 12.5 | .89† | 1.2 | 3.5 | 4.6 |
| Tetanus (11) | 8.02 ± .22 | 2.92 ± .22 | 1.52 ± .08 | 1.16 ± .08 | 1.90 ± .14 | .52 ± .04 |
| *t* | .79† | 6.9 | 4.2 | 4.2 | 3.4 | 4.6 |
| Meningitis (10) | 6.83 ± .29 | 2.28 ± .15 | 1.22 ± .09 | .95 ± .09 | 1.59 ± .22 | .79 ± .05 |
| *t* | 4.5 | 12.3 | 1.1† | 1.7† | .90† | 8.4 |

* Stand. error. † All values of *t* except these values are significant.
Figures in parentheses indicate No. of subjects studied.

*Results. Cholera*: β-globulin was high and was differentiated into $\beta_1$ and $\beta_2$ fractions. Fibrinogen increased. Total protein, albumin and α-globulin diminished. γ-globulin did not change. *Small pox*: Total protein and albumin diminished. No change in α- and β-globulins. Fibrinogen and γ-globulin increased and the latter was differentiated into $\gamma_1$ and $\gamma_2$ fractions. *Tetanus*: The different fractions of plasma globulins and fibrinogen increased. Albumin was low. Total plasma proteins did not change. *Meningitis*: The different fractions of globulins increased but values were not statistically significant. Total protein and albumin were low. Fibrinogen was high.

*Discussion. Cholera*: Diminution in plasma proteins might be partly due to hydremia as a result of saline transfusion, as average packed cell volume was 35%. It might also be due to disintegration of plasma proteins, as non protein N values were very high varying between 80 and 120 mg/100 cc blood. There might be possibility of decreased synthesis of albumin and α-globulin. High level of β-globulin in cholera might be due to stability of this protein complex by some mechanism as suggested by Tayeau(8). Elevation of fibrinogen in cholera is very significant. This might be due to dysfunction of the liver. The lowered albumin content might also indicate liver dysfunction(9,10). Antibodies are mainly contained in the γ-globulin of plasma proteins(11). As cholera is a very acute infectious disease and the disease is not prolonged, antibodies cannot find time to accumulate. This might explain the normal value of γ-globulin in cholera.

*Small pox*: Hypoproteinemia might be due to decreased synthesis of plasma proteins or due to its increased demand for formation of tissue protoplasm, which is continuously destroyed during the course of the disease. Fibrinogen is significantly raised in small pox possibly due to dysfunction of the liver. The increase in γ-globulin suggests formation of antibodies during the disease possibly by proliferation of lymphoid tissue(12,13). Antibody activity is more pronounced in $\gamma_1$ than in $\gamma_2$ fraction. Concentration of $\gamma_1$-globulin is nearly twice that of $\gamma_2$-globulin.

*Tetanus*: α-globulin is raised in pathological processes involving tissue destruction(14). Increased α-globulin in tetanus might also be due to increased tissue destruction. β-globulin increase might be due to stability of this protein complex(8). γ-globulin increase indicates increased antibody formation and increase in fibrinogen might be due to liver dysfunction.

*Meningitis*: It appears that in meningitis, the liver functions in an abnormal way as a result of which albumin decreases and fibrinogen is increased. The low albumin might also be due to albuminuria. Normal globulin

suggests that antibody formation does not proceed with the disease.

*Summary.* Different fractions of plasma proteins were determined by paper electrophoresis in 16 patients suffering from cholera, in 11 cases of tetanus, 10 cases of meningitis, 10 cases of small pox and 22 normal subjects. Total protein was lower than normal in all diseases studied except tetanus. Albumin was lower and fibrinogen was higher in all diseases as compared to normal values. Of the globulins, cholera cases had low $\alpha$, increased $\beta$ and normal $\gamma$-globulins. $\beta$-globulin contained $\beta_1$ and $\beta_2$ fractions not seen in normal persons. In patients suffering from small pox $\alpha$- and $\beta$-globulin fractions were normal but $\gamma$-globulin fraction which increased, contained $\gamma_1$- and $\gamma_2$-fractions. In patients suffering from meningitis and tetanus all the globulin fractions increased but in meningitis these increased values were not statistically significant. The implication of these changes has been discussed.

We are grateful to Dr. A. K. Datta Gupta, Superintendent, and to Dr. A. Mandal, Physician of Nilratan Sircar Medical College Hospitals, Calcutta, for kind permission to use patients of the hospital.

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Received January 11, 1957. P.S.E.B.M., 1957, v94.

## Cortisone and Mortality in Mouse Typhoid. I. Effect of Hormone Dosage and Time of Injection.* (22984)

SARANE THOMPSON BOWEN,† JOHN W. GOWEN, AND OSCAR E. TAUBER
*Departments of Genetics and Zoology, Iowa State College, Ames.*

The effect of cortisone on progress of bacterial infections remains a controversial issue. Some studies have indicated that this hormone lowers resistance(1,2). Other experiments have demonstrated that cortisone, in small quantities, will increase natural resistance to infection(3,4). This disagreement is of interest in regard to the *Salmonella* diseases, inasmuch as cortisone has been recommended as an adjunct in treatment(5). The following data summarize a study of the effects of cortisone on mortality of 4 strains of mice genetically differentiated for resistance to *Salmonella typhimurium.*

*Materials and methods.* Four inbred mouse strains from our Laboratory were used in this study: S, Z, K, and BALB/Gw, indicated as Ba. Each strain has been inbred by over 30 generations of brother-sister matings and, as a result, has become a distinct biotype with characteristic level of resistance to mouse typhoid. The wide range in resistance of these 4 strains may be seen in Table I. All mice were 5½-6½ months old. *Bacterial inocula-*

* This work supported in part by grant to Dr. John W. Gowen from Rockefeller Foundation. Journal Paper No. 3089 of Iowa Agr. Exp. Station, Ames.

† Present address: San Francisco State College, Calif.

TABLE I. Natural Resistance of Female Mice of 4 Strains to Intraperitoneal Inoculation of $2 \times 10^5$ Typhoid Bacteria (*Salmonella typhimurium* 11C).*

| Strain | Total inoculated | Survived | % survived |
|---|---|---|---|
| S | 1232 | 1173 | 95.2 |
| K | 534 | 337 | 63.1 |
| Z | 1440 | 616 | 42.8 |
| Ba | 897 | 2 | .2 |

* These data for mice from 100 to 400 days of age have accumulated over a period of years as part of results from numerous investigations.

*tions.* Mice were infected by intraperitoneal injection of approximately 200,000 bacteria of 11C strain of *S. typhimurium.* This 11C culture has been maintained by mass transfers on nutrient agar slants and has shown consistent virulence for 26 years. Eighteen hour cultures were suspended in 0.85% saline with 0.05 peptone. Suspensions were brought to standard density by appropriate dilutions within the linear portion of the calibration curve on a Lumetron photoelectric colorimeter. Each calibration was checked by plate count. The mean bacterial count in 26 inoculations was estimated to be 1.96 ± 0.04 hundred thousand cells. *Hormones.* Progesterone (Progestone Schering)‡ and desoxycorticosterone acetate (Cortate Schering) were used as crystalline suspensions in sesame oil. Control animals received an equal volume of Plain Sesame Oil (Schering). Cortisone acetate (Cortone Merck) was used as suspension in saline. Control animals in the cortisone experiments received an equal volume of Aqueous Vehicle No. 1 (Merck). Each injection was given in single subcutaneous dose. This injection preceded typhoid inoculation by 24 hours, unless otherwise noted. In all experiments, hormone-treated animals and their litter-mate controls were confined in the same cage; identification was by pedigree numbers.

*Results.* As little as 0.25 mg of cortisone reduced resistance of a mouse to *S. typhimurium.* When this quantity was given to males of the BALB/Gw strain, their mean survival time was reduced from 5.6 ± 0.1 to 4.9 ± 0.1 days. This difference is significant ($P < 0.01$). The observations on these 148 cortisone-treated mice and their litter-mate controls are presented in Table II. In a similar experiment on 56 litter-mate pairs of mice, a single injection of 0.01 mg of cortisone had no significant effect on resistance.

*Other steroid hormones.* Injections of 2 other physiologically active steroids had no effect upon typhoid resistance. Varying quantities of progesterone and desoxycorticosterone acetate were injected 24 hours before typhoid inoculation. Each hormone dose was given to 10 mice and their mortality was then compared with that of their 10 litter-mate controls. In doses of 0.1 mg and 1.0 mg, these 2 steroids did not significantly prolong survival of male mice of the BALB/Gw strain. In quantities of 1, 5, and 10 mg, neither hormone lowered typhoid resistance of male Z mice, a strain possessing intermediate resistance to typhoid infection. This indicates that massive doses of some steroid hormones do not lower resistance unless, like cortisone, they have a specific action in mouse typhoid.

*Large doses of cortisone.* In the following experiments the doses of cortisone ranged from 1 to 5 mg. Previous investigations had established that single doses to 5 mg were not toxic to normal mice(6,7). In our study, a 5 mg dose of cortisone had no significant effect on body weights of 6 mature male S mice when compared with their 6 litter-mate controls. Nevertheless, when smaller amounts (from 1 to 4 mg) were injected into S mice

TABLE II. Distribution of Deaths of Control and Cortisone-Treated Mice.*

| Exp. | | Day of infection 3 | 4 | 5 | 6 | 7 | 8 | 9 | Total |
|---|---|---|---|---|---|---|---|---|---|
| A | Control | 0 | 3 | 26 | 30 | 4 | 0 | 0 | 63 |
| | Treated | 0 | 12 | 34 | 16 | 1 | 0 | 0 | 63 |
| B | Control | 0 | 4 | 17 | 12 | 3 | 1 | 1 | 38 |
| | Treated | 3 | 19 | 12 | 3 | 1 | 0 | 0 | 38 |
| C | Control | 0 | 8 | 14 | 16 | 6 | 2 | 1 | 47 |
| | Treated | 1 | 17 | 15 | 12 | 2 | 0 | 0 | 47 |

* Mice were males of the Ba strain. Each treated mouse received 0.25 mg cortisone acetate one day before inoculation of *S. typhimurium.* Records for male Ba mice, tested over the same period as for females in Table I, show zero survival for 622 individuals.

‡ The authors wish to acknowledge generous supplies of hormones furnished by Merck and Co., Rahway, N. J., and by Schering Corp., Bloomfield, N. J.

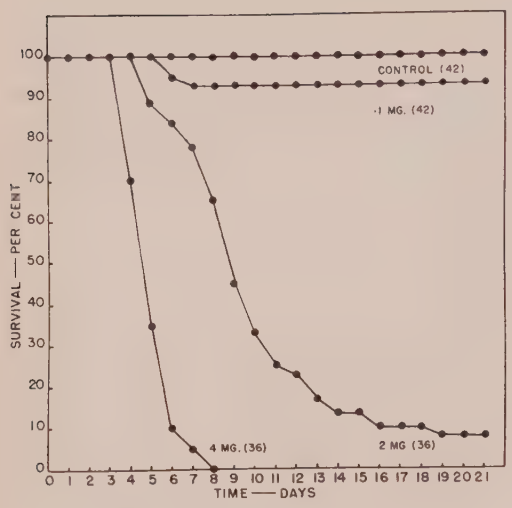


FIG. 1. Comparison of survival of control and cortisone-treated mice after inoculation with *S. typhimurium*. Figures in parentheses indicate No. of mice (S females) in each group.

which were inoculated with *S. typhimurium*, their resistance was markedly decreased. Mice of the S strain have a high resistance to typhoid. The effects of increasing doses of cortisone on mortality in mouse typhoid may be seen in Fig. 1. Twenty-one days after typhoid inoculation, none of the mice given 4 mg of cortisone had survived; 8% of those given 2 mg survived; 93% given 1 mg, and 100% receiving no cortisone had survived.

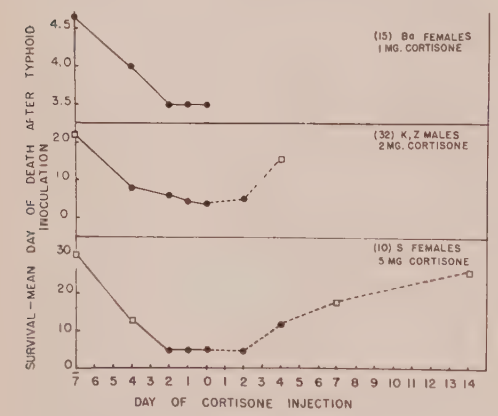


FIG. 2. Effect of single subcut. cortisone inj. given at varying times before or after inoculation with *S. typhimurium*. Experiments on 4 mouse strains (Ba, K, Z, and S) are illustrated. Numerals in parentheses indicate No. of mice used at each cortisone inj. represented by dot or square. Squares indicate that one or more mice survived beyond arbitrarily assigned day-of-death value (30th day of inj.). *Solid line*, cortisone inj. preceded typhoid inoculation. *Dotted line*, cortisone followed typhoid inoculation.

*Time of cortisone injection.* Cortisone had the greatest effect in reducing survival when administered within 2 days before or after typhoid inoculation. This may be seen in Fig. 2. In this experiment, equal numbers of mice were assigned at random to groups all of which were inoculated with *S. typhimurium* on the same day, but which received a single dose of cortisone either before or after typhoid inoculation. When cortisone was given after typhoid inoculation, there were 2 ways of calculating mean day-of-death-values. Length of survival could be measured from 1) time of typhoid inoculation, or 2) from time of cortisone injection. The former method was chosen for graphic presentation of data in Fig. 2. Because experiments on the 4 strains were done at different times, strain comparisons are not valid for this experiment. However, it is evident that each replication of this experiment, though on a different strain, is consistent: single injections of cortisone had the greatest effect in decreasing resistance when given within 2 days of typhoid inoculation.

*Discussion.* Other workers have shown that adrenal extracts have protected adrenalectomized animals from *Salmonella* endotoxins(8).§ Therefore, it is possible that the unfavorable action of cortisone on mouse typhoid resistance might be attributed to use of unphysiological doses. Robinson(3) has demonstrated that there is an optimal dose of cortisone which protects rats in *Diplococcus pneumoniae* infections, while greater amounts of hormone decrease their resistance. Nevertheless, one might expect that cortisone, when given in dosage which protects adrenalectomized mice from toxins, would increase resistance to infection. This is not the case. Delaunay and Lebrun found that 2 mg of cortisone protected adrenalectomized mice from *Salmonella* endotoxins. Yet in our study, less than 1 mg was effective in decreas-

§ Preliminary report by Delaunay and Lebrun in *Compt. Rend. Acad. Sci.*, 1951, v233, 1698.

ing resistance to infection. Because these small doses were shown to be non-toxic to the normal animal, it would seem that cortisone has some specific detrimental effect in mouse typhoid.

*Summary.* Cortisone, administered in single injections ranging from 0.25 to 5 mg, markedly decreased resistance of mice to *S. typhimurium*. Cortisone had the greatest effect on resistance when administered within 2 days before or after typhoid inoculation. Subcutaneous injections of two other steroids, progesterone and desoxycorticosterone acetate, in dosage range of 0.1 to 10 mg, had no effect on mortality in mouse typhoid.

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Received January 4, 1957. P.S.E.B.M., 1957, v94.

## Cortisone and Mortality in Mouse Typhoid. II. Effect of Environmental Temperature.* (22985)

SARANE THOMPSON BOWEN,† JOHN W. GOWEN, AND OSCAR E. TAUBER
*Departments of Genetics and Zoology, Iowa State College, Ames.*

In an investigation of effects of cortisone‡ on mice infected with *Salmonella typhimurium,* it was observed that during the hot summer months cortisone treatment was more effective in reducing survival. To determine the effect of environmental temperature on cortisone treatment, the following experiments were completed under conditions of controlled room temperature.

*Procedure.* Two mouse strains were used: an innately resistant strain (RI), and a strain (Z) of intermediate resistance to *S. typhimurium*. Of 280 untreated female RI mice, 100 to 400 days old, 77.9% survived a single dose of 200,000 11C *S. typhimurium,* whereas only 42.8% of 1440 Z females survived the same inocula. Evidence that this disease resistance is innate and not acquired has been reviewed by Gowen(1). Hormone-treated mice, 5½ to 7 months old, were given a single subcutaneous injection of cortisone acetate (Cortone, Merck) in saline with suspending agents (polyoxyethylene sorbitan monooleate and sodium carboxymethylcellulose) and benzyl alcohol as preservative. Control animals received an equal volume of Aqueous Vehicle No. 1 (Merck). Cortisone and aqueous vehicle injections were given 24 hours before typhoid inoculation. All mice were inoculated with $2X10^5$ viable cells of 11C strain of *S. typhimurium*. Throughout each 3 week experiment, cortisone-treated mice and their litter-mate controls were confined in the same cage; identification was by pedigree numbers. This investigation was conducted during the summer and an air conditioning unit was used to attain "moderate" temperature of 21° to

* This work was supported in part by grant to Dr. John W. Gowen from Rockefeller Foundation. Journal Paper No. 3090 of the Iowa Agr. Exp. Station, Ames.

† Present address: San Francisco State College, Calif.

‡ The authors wish to acknowledge generous supplies of cortisone furnished by Merck and Co., Rahway, N. J.

TABLE I. Survival of Control and Cortisone-Treated Mice in 2 Environmental Temperatures after Inoculation with *S. typhimurium*.*

| Exp. | | Z females | | | Z males | | | RI males | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Survived | % survived | Total | Survived | % survived | Total | Survived | % survived |
| | | Moderate environmental temp. (21° to 25°C)† | | | | | | | | |
| A | C‡ | 40 | 34 | 85 | 71 | 52 | 73 | | | |
| | T | 40 | 21 | 52 | 71 | 43 | 61 | | | |
| B | C | 34 | 23 | 68 | 30 | 15 | 50 | 52 | 45 | 86 |
| | T | 34 | 0 | 0 | 30 | 3 | 10 | 52 | 41 | 79 |
| | | Warm environmental temp. (29° to 36°C) | | | | | | | | |
| C | C | 26 | 18 | 69 | 22 | 10 | 45 | 45 | 33 | 73 |
| | T | 26 | 0 | 0 | 22 | 0 | 0 | 45 | 0 | 0 |
| D | C | 63 | 33 | 52 | | | | | | |
| | T | 63 | 0 | 0 | | | | | | |
| E | C | | | | 61 | 50 | 82 | | | |
| | T | | | | 61 | 0 | 0 | | | |

* Each treated mouse received subcut. inj. of Cortone (Merck) and each control received Aqueous Vehicle No. 1 (Merck) one day before intrap. inoculation of $2 \times 10^5$ living cells of *S. typhimurium*. Treated mice of Z strain were given 1 mg cortisone; treated mice of RI strain were given 2 mg cortisone.

† Temperature ranges were: Exp. A, 23°-25°C; Exp. B, 21°-24°C; Exp. C, 29°-36°C; Exp. D, 31°-36°C; and Exp. E, 29°-31°C.

‡ C = Control; T = Treated.

25°C (70° to 78°F) in the Isolation Room. When the air conditioner was not in operation, the temperature in the Isolation Room varied according to outside environment. This "warm" room ranged from 29° to 36°C (85° to 96°F). These temperature ranges are minimum and maximum values. Temperatures were noted 4 times daily during each 3 week experiment. Because only one room was available for these experiments, temperature comparisons were made on mice inoculated at different times. To compensate for this lack of experimental control, the 3 inoculations at "warm" temperatures were alternated with the two at "moderate" temperatures.

*Results.* The 5 successive bacterial inoculations (Exp. A through E) are summarized in Table I. Only comparisons of cortisone-treated mice and their litter-mate controls are valid here. Because temperature comparisons were necessarily made with animals which were inoculated at different times, certain experimental errors may have arisen. Nevertheless, 2 trends were consistent throughout these 5 independent experiments. First, treatment with cortisone reduced natural resistance of both the RI and Z mice. Second, number of survivors was further reduced if cortisone-treated mice were maintained at the warmer temperature (29° to 36°C). Not included in this Table are additional data accumulated during these 5 inoculations from 26 pairs of male Z mice, given 2 mg cortisone. At this dosage, the effect of temperature on survival was not significant. It is evident that the effect of environmental temperature may be seen only at a critical hormone dosage.

In general, survival of controls was also lower at warmer temperature. However, this difference in survival of controls is not significant. In previous observations in our Laboratory, more than a thousand mice of Z strain were inoculated with $2X10^5$ cells of *S. typhimurium* (11C) and survival was 42%. In the present study, the survival values for Z controls ranged from 45% to 85%. These figures were a bit higher, but not exceptionally so, considering the numbers of animals used in each experiment. Therefore we must conclude that environmental temperature had no significant effect on survival of infected controls.

Fig. 1 illustrates the combined survival data of Z females during 3 weeks following inoculation. When pooled data of replicates A and B were compared with those of repli-

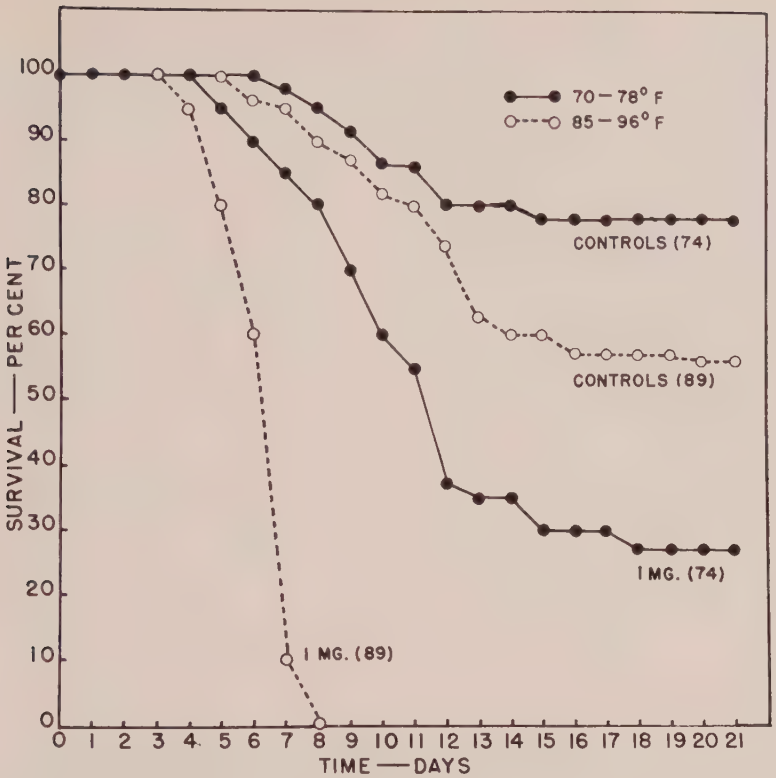


FIG. 1. Effect of environmental temperature on survival of control and cortisone-treated mice during mouse typhoid infection. Numbers in parentheses indicate No. of mice (Z females) in each group.

cates C, D, and E, the effect of temperature upon survival of cortisone-treated animals was evident by the fifth day after inoculation in both Z and RI strains.

*Discussion.* It has been shown that cortisone, in combination with its suspending agents and preservative, has a greater effect in reducing survival in mouse typhoid when the animals are maintained in a room temperature of 29° to 36°C than between 21° to 25°C. No data are available with which to evaluate the possible effect of the suspending agents and/or preservative. This lack makes insecure comparisons of survival of mice receiving Aqueous Vehicle with that of mice receiving no treatment at all. Scherr(2) has reported that in monilial infections, male mice treated with Aqueous Vehicle of cortisone showed higher mortality than untreated controls. This was not true of female mice he treated similarly. Scherr(3) has also shown that the toxic effect of cortisone for normal mice was greater at an environmental temperature of 35° to 37°C than at 25° to 29°C.

In studies of tourniquet shock, Moore(4) has shown that the mortality of injured mice is greater when maintained at temperature of 35°C instead of 25°C. Scherr(5) reported that mice infected with moniliasis have a higher death rate at 35° to 37°C than at 28° to 32°C. Therefore, it might be expected that mice injured by *Salmonella* endotoxins would show greater mortality in the warmer room temperature. In our study, however, infected mice, which did not receive cortisone, were not significantly harmed by maintenance at 29° to 36°C instead of 21° to 25°C. Moore(4) has reported that the mortality of tourniquet-injured mice maintained at 35°C was increased by high environmental humidity. In our experiments there was, however, no way of controlling humidity when air conditioner was not in use.

The reduced resistance, observed in in-

fected cortisone-treated mice in a warm environment, may be the result of the action of cortisone in decreasing capillary permeability (6). Also, it has been shown that cortisone blocks body temperature reducing effect of histamine(7). Weir(8) concluded that at moderate environmental temperatures (20°C) fever was not characteristic of mouse typhoid. He reported that rectal temperatures of infected mice were highly correlated with room temperature. Other studies have also shown that the mouse has little defense against increased environmental temperature(9,10). Hence, it is possible that the effect of temperature on toxicity of cortisone may be demonstrable only in the mouse.

*Summary.* Mice infected with *S. typhimurium* were maintained for 3 weeks either in a warm environmental temperature of 29° to 36°C or a moderate temperature 21° to 25°C. At the warmer temperature, subcutaneous injections of cortisone were more effective in reducing survival. Room temperature had no significant effect upon survival of those infected mice which received the Aqueous Vehicle of cortisone.

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Received January 4, 1957. P.S.E.B.M., 1957, v94.

## Cortisone and Mortality in Mouse Typhoid. III. Effect of Natural and Acquired Immunity.* (22986)

SARANE THOMPSON BOWEN,† JOHN W. GOWEN, AND OSCAR E. TAUBER
*Departments of Genetics and Zoology, Iowa State College, Ames.*

Cortisone may either increase or decrease resistance of infected animals, depending upon severity of the infection(1). Conversely, survival of animals infected by the same experimental inoculum may be either increased or decreased by cortisone treatment, depending upon hormone dosage(2). Most studies of hormone-infection relationship rely upon quantitative manipulation of either the hormone or the infectious inoculum. In the present investigation, a third experimental design was possible, by using host animals of varying genetic constitution. Seven strains of mice which differed in natural resistance to *Salmonella typhimurium* were given the same amount of cortisone and were inoculated with the same number of bacteria. It was then possible to establish the effect of cortisone on innate resistance to disease. The effect of cortisone on acquired resistance was determined by comparing mortality of normal and previously immunized mice during simultaneous treatment with cortisone and infection with *S. typhimurium*.

*Materials and methods.* All mice were between 5 and 7 months old. Seven inbred mouse strains were used: S, RI, Z, K, E, LGw, designated L, and BALB/Gw, designated Ba. These strains were chosen because of the wide range in their innate resistance to mouse typhoid. Inoculations of $2X10^5$ living bacteria of the 11C culture of *S. typhimurium* have established the following survival values: S, 95%; RI, 78%; K, 63%; Z, 43%; E,

* This work supported in part by grant to Dr. John W. Gowen from Rockefeller Foundation. Journal Paper No. 3091 of the Iowa Agr. Exp. Station, Ames, Project Nos. 1180 and 1187.

† Present address: San Francisco State College, Calif.

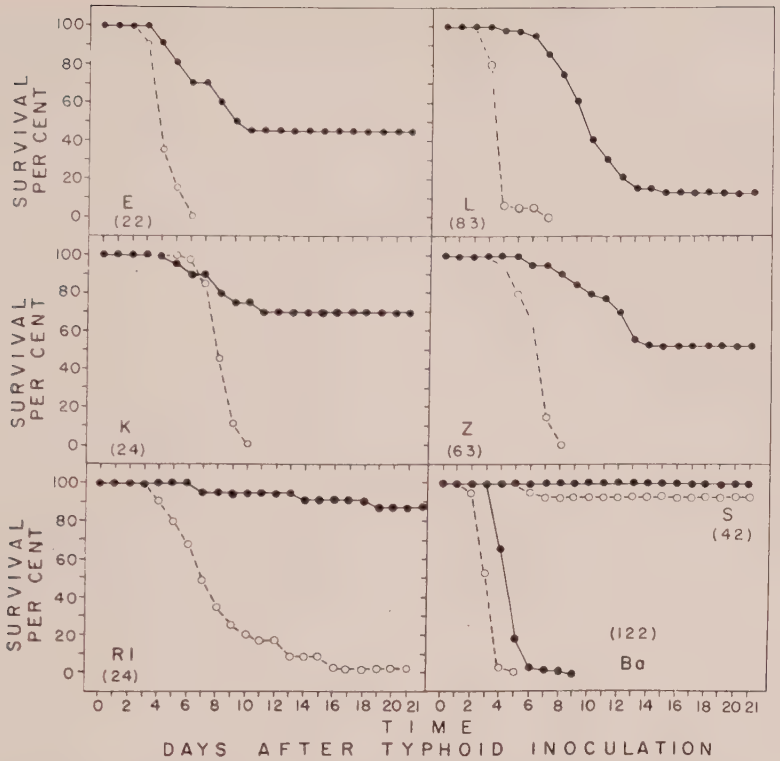


FIG. 1. Comparison of survival of control and cortisone-treated mice during mouse typhoid. The 7 mouse strains, ranked in order of decreasing resistance to *S. typhimurium*, are: S, RI, K, Z, E, L, and Ba. Numbers in parentheses indicate No. of mice in each strain which received 1 mg cortisone. An equal No. of animals was in the control group. *Solid line*, survival of controls. *Dotted line*, survival of cortisone-treated mice.

25%; L, 8%; and Ba, 0.2%. These earlier experiments utilized from 280 to 1440 female mice, 100 to 400 days old, in each strain. By testing resistance of progeny of double matings and reciprocal crosses, it has been rigorously proven that resistance to mouse typhoid of 3 of these strains (S, L, and Ba) is due to innate immunity rather than acquired immunity(4,5). The genetic basis of resistance is also demonstrated by the fact that for 15 years the breeding animals of all 7 strains have had no known contact with the pathogen, yet each strain maintains its characteristic level of resistance when tested with *S. typhimurium*(3). All mice were infected by intraperitoneal injection of $2X10^5$ living bacteria of the 11C strain of *S. typhimurium*. Treated mice were given a single subcutaneous injection of cortisone acetate (Cortone, Merck) in saline with suspending agents and benzyl alcohol as preservative. Controls were given equal volume of Aqueous Vehicle No. 1 (Merck).‡ Cortisone-treated mice and their litter-mate controls were kept in the same cage throughout the 3 week experiment. Each mouse carried its identifying pedigree number. The temperature in the isolation room ranged from 28° to 34°C.

*Results. Effect of natural resistance.* In the first experiment, 380 pairs of mature female mice were used. One member of each pair was given a subcutaneous injection of 1 mg cortisone, and its litter mate control was given an equal volume of Aqueous Vehicle. Twenty-four hours later, both mice were inoculated with *S. typhimurium*. In this way it was demonstrated that resistance of mice

‡ All cortisone was generously supplied by Merck and Co., Rahway, N. J.

in all 7 strains was decreased by pretreatment with cortisone. This may be seen in Fig. 1.

At the end of 3 weeks, survival of the most resistant strain, S, was reduced from 100% in controls to 93% in cortisone treated animals. In the other resistant strain (RI), survival was significantly reduced from 88% to 4% as a result of hormone administration. In 5 susceptible strains, 1 mg cortisone reduced survival to 0% within 10 days after inoculation of *S. typhimurium*. At the end of 3 weeks, survivals of controls of other strains were: K, 71%; Z, 52%; E, 45%; L, 12%; and Ba, 0%.

Differences in survival pattern of control and treated animals could be seen earlier in susceptible strains than in more resistant strains. Survival difference was seen on second day of infection in the Ba strain and on the third day in the E and L strains. In the 2 most resistant strains, the effect of cortisone was evident on the fourth day for the RI strain and on the sixth day for the S strain.

*Effect of acquired resistance.* Fifty sets of male mice from the most resistant S strain were used. Each set contained 3 litter-mates. One mouse in each trio had been immunized by intraperitoneal inoculation of $2X10^5$ living bacteria of the 11C culture of *S. typhimurium* 6 weeks previously. This immunized mouse and one of the normal mice were each given a single subcutaneous injection of 5 mg cortisone. The third member of each set was given an equal volume of Aqueous Vehicle. Twenty-four hours later, all 3 mice in each set were infected with $2X10^5$ cells of a viable 11C *S. typhimurium* culture. The results of this experiment are summarized in Fig. 2. Survival of immunized mice treated with cortisone was 98%. It is evident that this quantity of the infectious agent is not enough to kill either normal or immunized mice of the most resistant S strain. Infection has a high mortality rate in the S strain only when non-immunized animals are pretreated with cortisone. It had previously been determined that 5 mg cortisone is not toxic for uninfected mice (6). Hence, it has been demonstrated that survival of normal mice and of immunized infected mice is not significantly altered by a dose of cortisone which reduces survival of non-immunized infected mice from 100% to 0%.

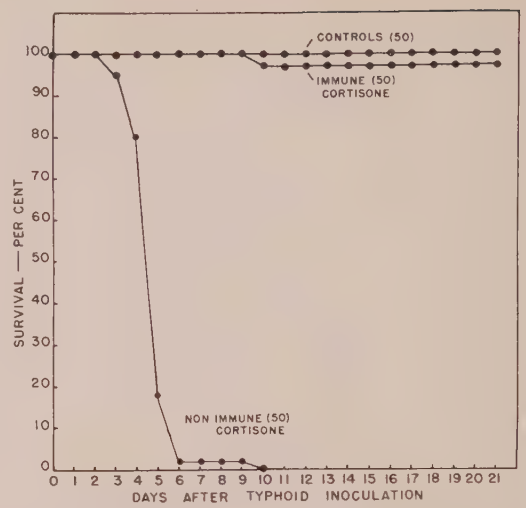


FIG. 2. Comparison of survival of normal and immunized male mice of S strain inj. with 5 mg cortisone one day before typhoid inoculation. Controls received an equal quantity of Aqueous Vehicle of cortisone. Figures in parentheses indicate No. of mice.

*Discussion.* Inbred strains of animals which differ in their innate resistance to a disease are an ideal material for the study of relationship between hormone dosage and severity of infection. The use of such strains obviates variation of amount of hormone or virulence of infectious agent. In studies in which dosage must be varied, there is a possibility that the effect of the hormone will be confounded with the effect of the suspending agent. This would certainly be true if the two had a synergistic action. It has been demonstrated that the cortisone vehicle reduces resistance of mice to monilial infections(1). In experiments in which the severity of the disease is controlled by gradations in the quantity of infectious agent, other problems of interpretation arise. The effect of a given quantity of hormone on a mild infection may represent an action upon bacterial invasiveness, whereas the effect of this same amount of hormone on a severe infection may represent an action upon resistance to toxins. Experiments which are based upon animals of varying natural resistance are free of these interactions.

Scherr has shown that cortisone increases resistance of mice to severe monilial infections, but decreases their resistance in mild infections(1). It is possible that a similar relationship holds true in mouse typhoid, but, if this were the case, one would expect that cortisone would be less harmful in the more susceptible strains. On the contrary, the detrimental action of cortisone showed itself earlier in the course of infection in susceptible strains.

Cortisone may have a more specific detrimental effect in mouse typhoid than in other diseases. It is well established that glucocorticoids and *Salmonella* infections both produce hyperglycemia, lymphopenia, and eosinopenia(7,8,9). Because cortisone administration simulates mouse typhoid in these respects, it might lower resistance through its action upon carbohydrate metabolism, or upon white blood cells. It is also possible that the level of cortical secretions may account for innate resistance or susceptibility of the strains. This concept has been investigated by Lurie *et al.*(10) in studies of tuberculosis in genetically resistant and susceptible rabbits.

We have shown that cortisone decreases natural immunity but not active acquired immunity to *S. typhimurium*. In studies of streptococcus infections in rabbits, Mogabgab and Thomas(11) also found that cortisone did not interfere with the mechanisms of active immunity. Payne *et al.*(12) have reported that both active and passive immunization protect cortisone-treated mice from *Pasteurella pestis* infections which are lethal for non-immunized mice.

*Summary.* Seven mouse strains which were selected for the wide range in their natural resistance to *S. typhimurium* were used. Typhoid resistance of every strain was decreased when 1 mg cortisone was given one day before typhoid inoculation. The difference between survival of cortisone treated mice and that of controls was evident at an earlier time in the genetically susceptible strains than in resistant strains. A single injection of 5 mg cortisone which reduced survival of non-immunized mice from 100% to 0% had no significant effect on survival of immunized mice.

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Received January 4, 1957. P.S.E.B.M., 1957, v94.

# Variations in Paper Electrophoretic Patterns of Dog Sera and Plasmas Prepared by Different Technics.* (22987)

WILLIAM H. GOLDWATER AND CECIL ENTENMAN
*Biological and Medical Sciences Division, U. S. Naval Radiological Defense Laboratory, San Francisco, Calif.*

Recent paper electrophoresis studies on the sera of X-irradiated dogs afforded the occasion to compare the results obtained with those of other investigators on the electrophoretic patterns of normal dog plasma(1-3) and serum(4-8). Apart from the less precise designations for the protein fractions used by some of these workers, a distinct, but previously unreported difference between serum and plasma electrophoresis patterns was apparent, in that the alpha-4 globulin seemed to predominate over the alpha-3 component in dog sera, while the reverse tended to hold for dog plasmas.

A distinct exception to these findings has been reported(5). However, the present work describes how the patterns obtained may depend on technics of collection for different types of serum or different types of plasma, thus accounting for this apparent exception. This communication describes further the nature of these "new" protein differences between dog sera and plasmas.

*Methods.* Normal female mongrel dogs weighing 15-35 lb were maintained and postabsorptive blood samples were drawn as described earlier(9). Samples designated *"standard serum"* were obtained by our customary technic, allowing blood to clot one hour at room temperature and then in the refrigerator for another hour, after which centrifugation yielded the "standard serum." *"Sedimented serum"* was obtained similarly, except that the whole blood was centrifuged immediately upon collection, so that clotting took place after the blood cells had been sedimented to the bottom of the tube. Clotting at room temperature, refrigeration, and centrifugation, as above, then yielded the "sedimented serum." *Oxalated plasma* was obtained from blood samples collected in tubes containing 1.2 mg ammonium oxalate and 0.8 mg potassium oxalate per ml blood(10), and *heparinized plasma* was obtained from blood collected in heparinized syringes. All samples were finally obtained by centrifugation at 2000 rpm (about 1000 x g), and were handled together at room temperature and during refrigeration and centrifugation. Paper electrophoresis was performed as described previously, using horizontal migration in a closed, refrigerated cabinet, on Whatman 3 MM filter paper and barbital buffer at pH 8.6 and ionic strength 0.05. Five microliter samples of serum or plasma were pipetted to spots on the starting line, midway between the buffer vessels, and constant voltage was applied for 6½ hours at 500 volts, or 16½ hours at 175 volts. At the end of the run the paper was dried for 20 minutes in a forced-draft oven at 60°C. The position of a small tan spot was marked at the edge of the paper. This location is the same as that of the alpha-4 globulin in the subsequently developed pattern from normal dog serum, although it is absent or, at most, very faint after runs on dog plasma. Protein patterns were stained with bromphenol blue according to Piantanida and Muic(11), lipids with Sudan Black B by the method of Swahn(12), and polysaccharides following the technic of Köiw and Grönwall(13).

*Results.* In Fig. 1 and 2 are shown the patterns obtained by staining proteins and polysaccharides, respectively, on samples of "standard serum," "sedimented serum," oxalated plasma, and heparinized plasma from the blood of a normal dog. The results shown are typical of those obtained in similar comparison runs on over 15 dogs, sampled in the normal, fasting state.

* This work was supported, in part, by funds provided by Bureau of Medicine and Surgery, U. S. Navy Department.

The opinions or assertions contained herein are private ones of the authors and are not to be construed as official, or reflecting the views of the Navy Department.

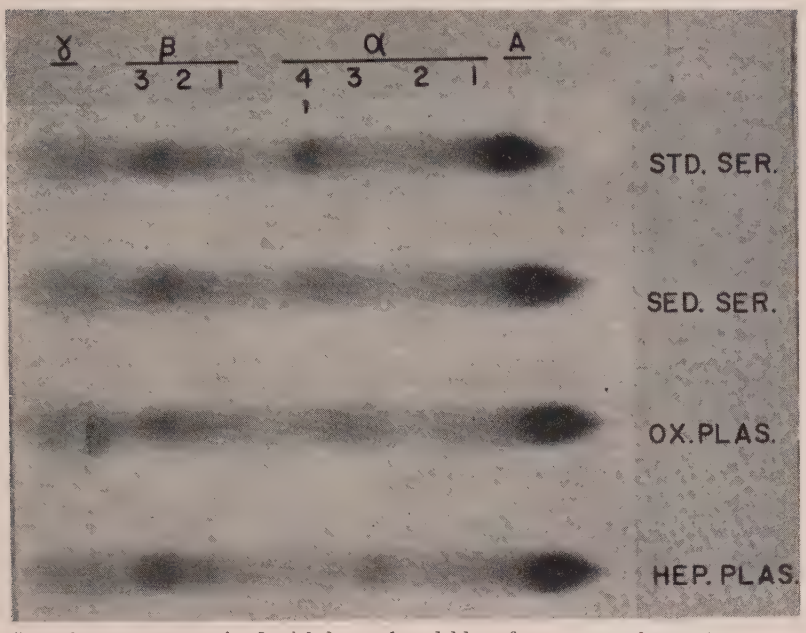


FIG. 1. Protein patterns, stained with bromphenol blue, from paper electrophoresis of samples of normal dog "standard serum," "sedimented serum," oxalated plasma, and heparinized plasma, reading from top to bottom. Starting line toward left, migration is from left to right. Electrophoretic components labeled at top are albumin, $\alpha$-1-2-3-4, $\beta$-1-2-3, and $\gamma$-globulin. Pencil mark, locating "small tan spot" described in text, is directly under "4" in $\alpha$-globulin region.

The "standard serum" protein pattern (Fig. 1) is seen to include on the right hand side the prominent albumin spot, on the trailing side of which a slight tail represents the alpha-1 globulin. Following this are faint alpha-2 and -3 globulins, and then a more prominent alpha-4 globulin spot. Three beta globulin spots may be distinguished, after which comes the broad gamma globulin spot, which has migrated backwards due to electröosmosis(14).

The *"sedimented serum"* pattern differs slightly in having a somewhat fainter alpha-4 spot and a stronger alpha-3 globulin. Similar patterns were obtained with *oxalated plasma*. However, with *heparinized plasma* hardly any stain appears in the alpha-4 position, but a pronounced spot is apparent in the alpha-3 globulin. As was to be expected, both oxalated and heparinized plasmas show staining of the fibrinogen fraction at the starting line, although this component was rather weak in the case of the heparinized plasma shown here in Fig. 1.

Patterns treated with the polysaccharide stain are shown in Fig. 2. The *"standard serum"* pattern shows very faint staining in the albumin region, no stain in the alpha-1 globulin, faint spots in the alpha-2 and -3 positions, a more prominent alpha-4 component, and additional faint spots in the beta globulin regions. As in the case of the protein patterns, the *"sedimented serum"* and oxalated plasma patterns show a more even distribution of stain between the alpha-3 and alpha-4 positions. The *heparinized plasma* again shows the stain concentrated in the alpha-3 globulin.

Similar differences in lipid patterns from corresponding sera and plasmas have not been observed. However even if such differences were present, they would be difficult to detect due to the faint staining of the small quantities of lipids in the alpha-2-4 region of the electrophoretic patterns of normal dog serum or plasma.

*Discussion.* The results described here show that a distinct difference exists between the electrophoretic patterns obtained from dog serum and plasma, apart from the an-

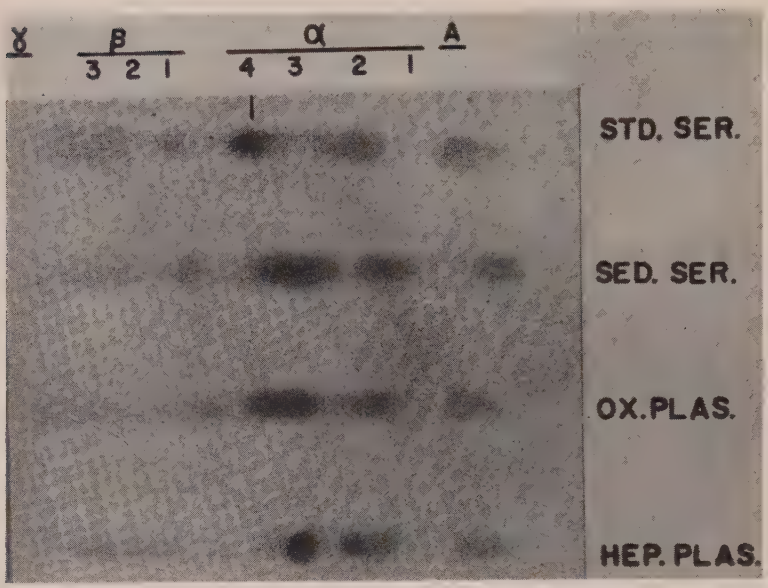


FIG. 2. Polysaccharide patterns, stained with periodic acid-fuchsin sulfite, from paper electrophoresis of samples of normal dog serum, "sedimented serum," oxalated plasma, and heparinized plasma, from top to bottom. Other details as in Fig. 1.

ticipated presence of fibrinogen in the latter. An additional observation has been that technics of obtaining different types of sera or different types of plasmas can also produce changes in the electrophoretic patterns. Of special significance in the interpretation of either free or paper electrophoresis patterns from the dog is the finding that the pattern shifts observed are a function of polysaccharide components as well as of proteins, thus implicating glycoproteins in the phenomenon. The two extremes of this situation are represented by the "standard serum," with the prominent alpha-4 globulin in protein and polysaccharide patterns, and the heparinized plasma, with the alpha-4 globulin greatly diminished and the alpha-3 component predominant in both protein and polysaccharide patterns.

Although the shift to a faster mobility resembles that seen in proteins of stored human plasma(15) the freshness and equal age of all samples compared in the present study eliminate ageing as an explanation for this phenomenon. Nor is the effect explainable solely as an *in vitro* heparin effect(16,17), since it is seen to a similar degree in oxalated plasma and "sedimented serum," the latter containing no anticoagulant whatsoever. Comparison of results in these two cases, one unclotted, the other clotted, eliminates the blood clotting mechanism as the sole cause for the shift from alpha-3 to alpha-4.

*Summary.* The alpha globulin portions of the electrophoretic patterns of dog sera and plasma have been shown to be affected by the technics of preparation. While "standard serum" patterns show a predominance of alpha-4 over alpha-3 protein and polysaccharide, "sedimented serum" and oxalated or heparinized plasma show greater proportions of alpha-3 components.

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Received October 1, 1956. P.S.E.B.M., 1957, v94.

## Myelograms Relating to Anemia and Hematopoiesis Following Thermal Injury in Rats*. (22988)

MILES D. MCCARTHY AND JOHN ODELL

*Department of Zoology, Pomona College, Claremont, Calif.*

Anemias following thermal injury in man and laboratory animals have been repeatedly observed(1-6) and have been shown in part to result from increased rate of hemolysis(1, 2,5) and disturbed liver function(4). Suppressed medullary erythropoiesis has also been suggested as contributing to the anemia by James and co-workers(2) while Moore and his co-workers(1) and Wintrobe and his co-workers(7) have presented data from radioiron uptake studies that indicate direct medullary inhibition. However, the work of Davis, Alpen and Davis(5) and Davis, Alpen and Sheline(6) showed an increase in rate of radioiron uptake which they interpreted as evidence for increased extramedullary and medullary erythropoiesis.

In this investigation we have studied the quantitative changes in femoral marrow of control rats, bled rats and thermally injured rats. We have attempted to discover from the data (a) whether suppression of the medullary hematopoiesis exists following thermal injury and (b) whether there is any quantitative difference in medullary hematopoiesis between moderately injured animals and severely injured animals.

*Materials and methods.* Unshaved male Wistar rats weighing between 200 and 210 g were used. Rats were housed individually in wire mesh cages at room temperature varying between 72 and 78°F and relative humidity varying between 20 and 40%. Except for the immediate 10 hour period following injury, all rats received Purina Laboratory Chow *ad lib.* This diet was supplemented once a week with carrots. Groups A and B received thermal injuries of 50 ± 2% and 20 ± 2% of the body surface respectively. Group H was bled and infused. Group C was the uninjured control. Thermal injury was effected under ether anesthesia in water at 90°C for 35 seconds. Prior to experiment a polyethylene tube was inserted into the posterior facial vein of each rat. Immediately following the thermal injury each rat was infused with 4% body weight of 1/6 M sodium lactate solution and 14% body weight of 1.4% sodium chloride solution. The details of these procedures have been previously reported(8). Animals in control group H were operated, bled by cardiac puncture and infused as described above. Three cc of blood were withdrawn to approximate immediate blood loss from thermal injury. This volume was determined from our $Fe^{59}$ studies. In each of the thermally injured groups A and B and in the bled group, H, 3 animals were sacrificed for femoral bone marrow studies at 24, 48, 96, 168 hours and 2 and 4 weeks following injury or bleeding re-

* This investigation was conducted under contract from Office of Surgeon General, Department of Army.

spectively. Three animals were sacrificed at 8 weeks in groups B and H. The group A experiment was terminated at 4 weeks after injury due to difficulties of keeping such severely injured animals alive. In the uninjured control group C, 3 animals were sacrificed at each of the following intervals from initial starting date of experiment: 1, 4, and 8 weeks. Our method of bone marrow studies was in part suggested from experiments of Fruhman and Gordon(9). The rats were killed with ether and both femurs immediately removed and trimmed clean of surrounding tissue. Each femur was weighed before and after demedullation and average weight differences in mg were recorded as marrow weight. After initial weighing both femurs were carefully cracked and samples of marrow were drawn into certified erythrocyte pipettes to the 0,2 level. Six such pipettes were prepared. To 3 of these Hayem's solution was added and to the other 3 the standard acetic acid-gentian violet white cell diluent was added. The diluent in all pipettes was added to the 101 mark. After the pipettes were shaken for 3 minutes, certified hemacytometers were charged. Cells were counted in the 4 corner and center squares of the centrally ruled 1 mm square. The average of 6 counts, 2 from each pipette, was determined for total leukocyte and erythrocyte values recorded. Immediately after taking the marrow samples, several marrow smears were made by the imprint smear technic. These smears were fixed with osmic acid vapor and stained with Wright-Giemsa. From the smear preparations, 1000 leukocytes were identified and classified based upon the descriptions given by Endicott and Ott(10) into the following classes: a. immature neutrophilic granulocytes; b. mature neutrophilic granulocytes; c. lymphocytes; and d. other cells. Each class of cells was expressed in terms of cells per $mm^3$ by multiplying the total leukocyte count by the decimal fraction of each particular class.

*Results. Marrow weights.* Average marrow weights for all groups are shown in Fig. 1. Uninjured controls, group C, showed progressive increase with age. In bled group H,

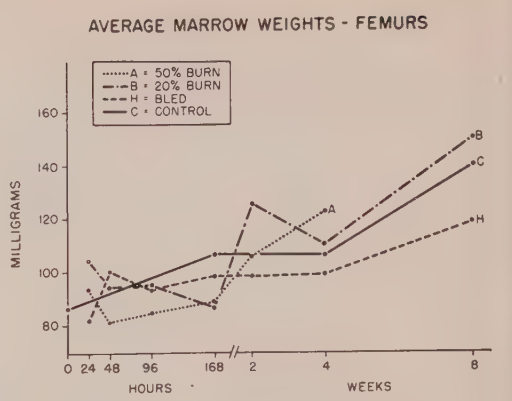


FIG. 1. Avg wt of marrow from right and left femurs in mg determined from differences in femur wt before and after demedullation. Each point on each curve represents avg values for 3 rats.

a sharp increase occurred from 24 to 48 hours after bleeding followed by a slightly lower value at 96 hours. From 96 hours after bleeding a progressive increase was observed. The animals given 50% thermal injuries showed a decrease at 48 hours after injury and then a progressive increase. The animals given 20% thermal injuries showed increased marrow weights at 24 hours after injury which was followed by a progressive depression throughout the first week. Then there occurred a sharp increase at 2 weeks after injury. Subsequent values paralleled those of the uninjured controls. *Erythrocytes.* Average erythrocyte counts are shown in Fig. 2. In uninjured controls there occurred an increase of erythrocytes with age. Bled con-

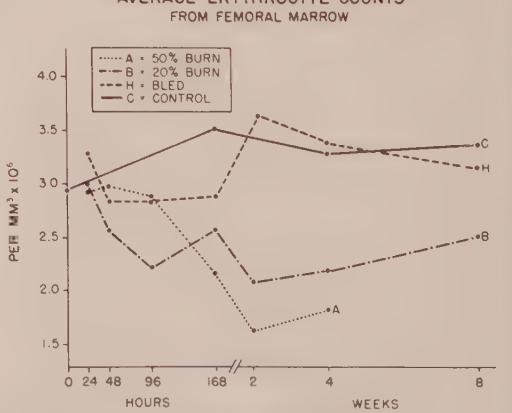


FIG. 2. Determined from hemacytometer counts of diluted femoral marrow. Each point on each curve represents avg values for 3 rats.

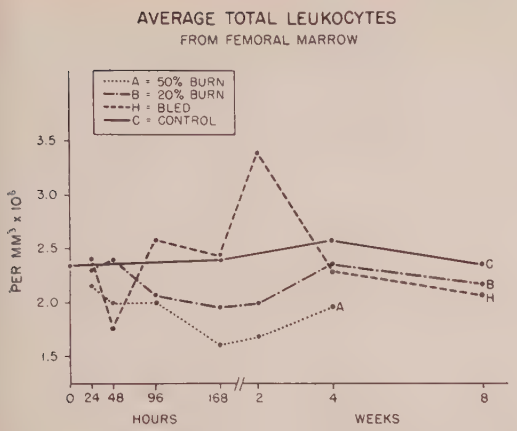


FIG. 3. Determined from hemacytometer counts of diluted femoral marrow. Each point on each curve represents avg values for 3 rats.

trols showed a decrease in erythrocytes during the first week after bleeding following which their erythrocyte numbers were not significantly different from uninjured controls. The animals given 20% thermal injuries showed a progressive depression through the first 96 hours, a recovery to the 48 hour level at 1 week after injury, then a secondary depression at 2 weeks resulting in numbers below the 96 hour post-injury level. From 2 weeks through 8 weeks their erythrocyte level progressively increased toward the pre-injury level. The animals given 50% thermal injuries showed a precipitous decline in erythrocyte numbers from 96 hours through 2 weeks after injury and at the 2 week interval reached a level approximately 400,000 cells less than the corresponding value for the animals given 20% injuries. At 4 weeks after injury the erythrocyte numbers of the animals given 50% thermal injuries increased by approximately 150,000 cells.

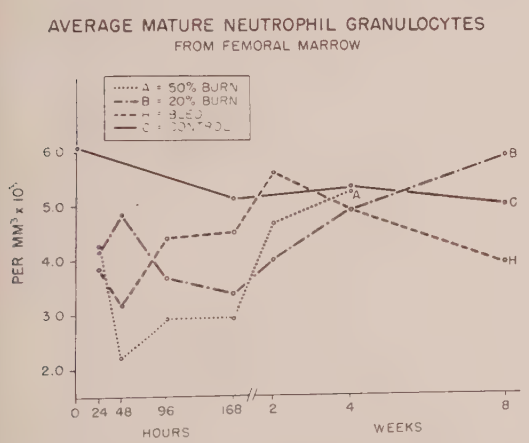


FIG. 4. Determined from smears by multiplying total leukocyte counts from femoral marrow by decimal fraction of these cells out of 1000 classified leukocytes. Each point on each curve represents avg values for 3 rats.

*Leukocytes.* Since the changes in the immature neutrophilic (n.) granulocytes were found to parallel the changes in the total leukocytes, (Fig. 3) the former have not been presented here. The uninjured control animals, group C, maintained approximately the initial levels of total leukocytes and of mature n. granulocytes, (Fig. 4), throughout the experimental period. The bled animals showed a depression of the numbers of total leukocytes and mature n. granulocytes during the first 48 hours after bleeding following which they progressively recovered and attained levels well above the controls by the second week. From the fourth week through the eighth week after bleeding the numbers of these cells approximated those of the control group C. In the animals given 20% thermal injuries the total leukocytes and mature n. granulocytes decreased from 48 hours through 1 week following injury. Then they increased through 4 weeks after which the total leukocytes leveled off parallelling the uninjured control and bled groups. The mature n. granulocyte numbers of the 20% injured animals were greater than those of the uninjured controls at 8 weeks following injury. The 50% thermally injured group showed a precipitous reduction in the numbers of mature n. granulocytes during the first 48 hours after injury. Then their numbers increased through 4 weeks at which time they reached the control level. The total leukocytes of the 50% injured group declined through the first week following injury. At 4 weeks the numbers of these cells in this group were considerably less than those in any of the other groups.

*Discussion.* The thermally injured rats studied in this investigation differ from similarly injured patients in the therapeutic procedures necessary for survival. Following the primary shock period no intravenous

fluids were given and no antibiotics were administered. The skin over the burned area remained in place for 3 to 4 weeks following the injury. The area under the injury became hard and dry within 24 to 48 hours following sloughing. Thus in our judgment, the quantitative changes observed in the bone marrow, reflecting either a stimulative or a suppressive effect, would be uncomplicated by the excessive loss of fluid and blood cells from granulating wounds, or excision and grafting procedures.

Our data show a clear-cut difference in the marrow erythrocyte populations between the burned rats and the bled and uninjured controls. The bled animals after the first week following bleeding showed a definite marrow stimulation which resulted in erythrocyte levels comparable to the uninjured controls. Both thermally injured groups of animals showed decreased numbers of erythrocytes following the first week after injury which we interpret as a suppression of marrow function. This interpretation is strengthened by our observations of erythrocyte counts in the peripheral circulation of similarly injured animals(11). We found an approximate reticulocyte increase of only 3% above the preinjury level at 2 weeks after injury. In addition no significant increase in the number of nucleated erythrocytes was found. The reduction in marrow erythrocyte numbers in the injured animals was directly related to the severity of the injury. Following the 1 week post-injury interval, the numbers of marrow erythrocytes were significantly lower in the animals given 50% body surface thermal injury. Further evidence of a suppression of medullary hematopoiesis was found when the total leukocyte counts of the bled and injured rats were compared. From the first through the second week after bleeding a tremendous increase in the medullary total leukocytes occurred while only a very slight increase in the numbers of these cells occurred in the two thermally injured groups. Also it was found that the numbers of the medullary total leukocytes in the injured animals were lower in the more severely injured. The numbers of the medullary immature granulocytes which are a measure of the hematopoietic activity were observed to be suppressed from 96 hours through 2 weeks after injury when compared to those at similar intervals in the bled animals. Again it was found that the numbers of n. granulocytes were significantly lower in the more severely injured. Thus our data support the conclusions drawn from radioiron uptake studies by Moore *et al.*(1) and Wintrobe *et al.*(7) that suppression of marrow function is definitely a contributing cause of anemia after thermal injury. Our data would not support the conclusions of Davis *et al.*(5,6) drawn from radioiron uptake studies in thermally injured rats that increased medullary hematopoiesis occurs following injury.

*Summary.* Marrow weights and the numbers of cells per $mm^3$ in femoral marrow of erythrocytes, total leukocytes, immature and mature n. granulocytes were obtained in uninjured rats, bled rats, and rats given thermal injuries of 20% and 50% body surface. Significantly lower numbers of erythrocytes, total leukocytes and n. granulocytes were found in the thermally injured rats during first two weeks following injury compared to the numbers of these cells occurring at similar intervals in the uninjured controls and bled animals. The numbers of these cells following thermal injury were found to be inversely related to the percentage of body surface injured. These data support the conclusion that a suppressed medullary hematopoiesis occurs after thermal injury and is partially responsible for the circulatory anemia found in thermally injured animals.

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Received October 29, 1956. P.S.E.B.M., 1957, v94.

## Effect of Temperature on Ionographic Mobilities in Paper Stabilized Media.* (22989)

HUGH J. MCDONALD, JAMES L. FORESMAN† AND EDWARD W. BERMES, JR.‡

*Department of Biochemistry, Graduate School and Stritch School of Medicine, Loyola University, Chicago, Ill.*

In a recent article on paper electrophoresis (1), Forbes and Taylor reported that although the rate of electromigration of beta lipoproteins was considerably less at 5°C than at room temperature or at 35°C, no effect of temperature was evident in lipoproteins migrating with the albumin, alpha$_1$ globulin. They also reported that rate of movement of the main serum components was apparently the same at different temperatures. The non-uniformity of the effect of temperature on mobilities of the substances studied by them was in such marked contrast to those observed in this laboratory(2,3) that it was decided to reinvestigate the effect of temperature on ionographic mobilities in paper stabilized media for a range of substances varying from low molecular weight materials, such as a simple dye, to more complex substances such as plasma proteins and lipoproteins.

*Methods and material.* The Precision Ionograph,§ which utilizes the horizontal paper strip in a water-saturated atmosphere(4), was used with Whatman No. 1 filter paper strips (0.5 inch in width and 40-45 cm in length) and a veronal buffer of pH 8.6 and ionic strength of 0.03 and 0.01. The apparatus permits use of 7 paper strips simultaneously. In nearly all cases, the runs on each migrant were made at 4 different temperatures, namely 25°C, 15°C, 9°C and 4°C. During each run the temperature was maintained at ± 0.2°C by circulating liquid of constant temperature through the double-walled shell of the ionographic apparatus. The substances whose mobilities were determined included bromphenol blue (Hartman-Leddon Co., Philadelphia), crystalline bovine plasma albumin (Armour Laboratories, Chicago), and the following serum fractions, obtained from fresh pooled human serum, namely, albumin, alpha lipoprotein, alpha globulin, beta lipoprotein and gamma globulin. The veronal buffer having an ionic strength of 0.01 was employed for the bromphenol blue and bovine plasma albumin, while an ionic strength of 0.03 was used for all determinations on human serum fractions. The average time of a run was 3-4 hours, and a potential gradient of 4-5 volts/cm was used, maintained constant during the run. For the runs at 25°C helium was introduced into the water-saturated atmosphere while at lower temperatures, the water-saturated atmosphere alone was employed. Equilibration time, *i.e.*, the time the paper strips were permitted to stand, after wetting and with the current on, to reach a constant value for the ratio of "weight of buffer solution" to "weight of paper" was determined for the particular paper and buffer used, before beginning the runs.

* Supported in part by grant from Chicago Heart Assn.

† Medical Student Part-time Research Fellowship, 1955-56, by Division of Research Grants, N.I.H., U.S.P.H.S., Dept. of Health, Washington, D.C.

‡ Standard Oil Foundation Research Fellow; supported by Standard Oil Co. (Ind.).

§ Precision Scientific Co., Chicago, Ill.

TABLE I. Ionographic Mobilities of 7 Different Substances as a Function of Temperature.

| Migrant | Temp., °C | Mobility, $\mu$/sec./v/cm | Stand. dev. | No. of runs |
|---|---|---|---|---|
| Bromphenol blue | 25 | 1.21 | .03 | 4 |
| | 15 | .917 | .03 | 1* |
| | 9 | .773 | .01 | 2 |
| | 4 | .685 | .06 | 2 |
| Bovine plasma albumin | 25 | .884 | .14 | 5 |
| | 9 | .688 | .03 | 2 |
| | 4 | .553 | .02 | 2 |
| Human serum albumin | 25 | .725 | .04 | 4 |
| | 15 | .599 | .04 | 1* |
| | 9 | .545 | .04 | 5 |
| | 4 | .491 | .03 | 5 |
| $\alpha_2$ globulin | 25 | .448 | .03 | 4 |
| | 15 | .374 | .03 | 1* |
| | 9 | .339 | .03 | 5 |
| | 4 | .307 | .03 | 4 |
| $\gamma$ globulins | 25 | .245 | .04 | 4 |
| | 15 | .206 | .03 | 1* |
| | 9 | .170 | .03 | 5 |
| | 4 | .174 | .02 | 4 |
| $\alpha$ lipoproteins | 25 | .733 | .05 | 4 |
| | 15 | .564 | .06 | 1* |
| | 9 | .509 | .07 | 5 |
| $\beta$ lipoproteins† | 25 | .225 | | 1 |
| | 15 | .173 | | 1 |
| | 4 | .132 | | 1 |

* Since only one run was made at 15°C, the stand. dev. was calculated by taking avg of stand. dev. at other temperatures.

† Figures for mobility represent avg of group of 7 ionograms run simultaneously.

The ratio was constant at a value of 1.58, after the strips were equilibrated for 30 minutes. It was also determined that movement of the migrant was a linear function of time and of the potential gradient, respectively, under otherwise constant experimental conditions. These are fundamental criteria which must be met if mobility measurements are to be meaningful(4,5). Movement of a particular component, for determining its mobility, was taken as the distance between forward or leading edge of a given band and the forward edge of initial narrow streak of the material which was applied initially to the paper strip at point of origin. The protein fractions were stained with bromphenol blue, while Sudan black B was used to develop lipoprotein ionograms.

*Results.* As pointed out in an earlier publication(3), the electromigration of a charged particle through the buffer solution saturating the paper strip, is fundamentally a simple rate process and it would therefore be expected to increase with increasing temperature. It would moreover be expected to exhibit the usual temperature dependency of such processes(6), namely, that when the natural logarithm of mobility is plotted as a function of the reciprocal of the absolute temperature, a straight line would result. Mobilities of various substances studied are listed in Table I. When these were plotted in the manner described above, the results were as shown in Fig. 1 and 2.

Each experiment consisted of 7 simultaneous runs. Figures in the mobility column of Table I represent averages, *e.g.*, for human serum albumin; 0.725 represents the average from 28 individual ionograms, run in 4 groups of 7. The standard deviation is based on the number of "groups of 7 strips," rather than on the number of individual ionograms. It must be borne in mind that the value for standard deviation is accentuated by this technic, and would be much smaller, numerically, if based on the number of individual ionograms. The reason for using the above

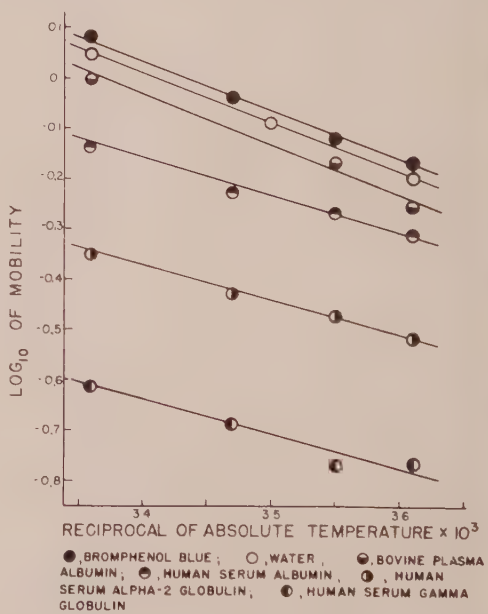


FIG. 1. Effect of temperature on ionographic mobility of bromphenol blue, on plasma protein fractions, and on coefficient of viscosity of water. The ordinate scale, for water, represents logarithm of reciprocal of coefficient of viscosity.

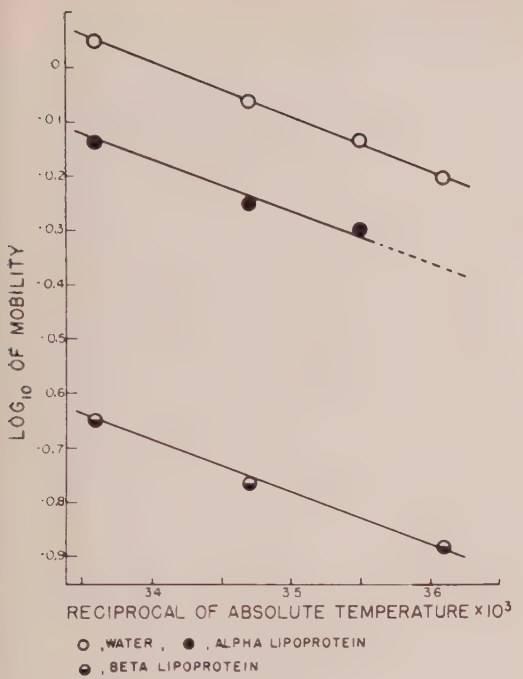


FIG. 2. Effect of temperature on ionographic mobility of human plasma lipoproteins. The ordinate scale, for water, represents logarithm of reciprocal of coefficient of viscosity.

procedure is that any human error, such as a wrong voltage reading, would cause a given group of 7 ionograms all to be in error by the same amount.

Since the normal increase in mobility of a migrant with increase in temperature would parallel the increase in fluidity of water with the same temperature increase, it would be expected that if the "logarithm of the reciprocal of the coefficient of viscosity of water" is plotted as a function of the "reciprocal of the absolute temperature," a straight line should be obtained. This linearity is shown in Fig. 1 and 2.

In the case of the beta lipoproteins, erratic mobility results were obtained in the earlier experiments. In general, as a serum sample aged, under refrigeration at 4°C, the mobility values dropped. This confirms the observations made earlier by Gottfried *et al.*(7). When, however, mobilities at 25°C, 9°C, and 4°C, were determined by running the experiments simultaneously, but using 3 separate ionographic apparatuses, the results were consistent with the behavior observed for all other migrants.

Since the effect of temperature on mobilities of all substances studied was nearly the same, it is possible to represent temperature dependency of a wide variety of migrants, under otherwise constant experimental conditions, by the following simple equation.

$$\ln u = \frac{m}{T} + A$$

where u = mobility of migrant in some definite system of units, ln = natural logarithm, T = temperature in degrees Kelvin, m = slope of line, for water itself, and is the value to be used for all other substances where the lines parallel that of water, and A is a constant which is characteristic for each migrant. Converting to logarithms to base 10 the equation becomes:

$$\log u = \frac{2.303 \times m}{T} + 2.303\ A$$

or

$$\log u = \frac{2.303 \times m}{T} + B$$

For a given migrant, then, the mobilities at all temperatures in the range 4°C to 25°C can be computed from a value determined at any one temperature within this range. As an example, consider the case of bromphenol blue. At 25°C, the mobility was 1.21 $\mu$/sec. per volt/cm. Insertion of the proper values in the equation then yields:

$$\log 1.21 = \frac{-980}{298} + B$$

Where –980 represents the slope of the lines shown in Fig. 1, which parallel that for water itself, and is therefore applicable to the case of bromphenol blue. From the equation the constant B is determined to be 3.37. The mobility at 4°C, then, can be computed as follows:

$$\log u_{4°C} = \frac{-980}{277} + 3.37$$

whence u is 0.681 $\mu$/sec. per volt/cm. The experimental value at that temperature is listed in Table I as 0.685.

Since it is now generally conceded that no single electroosmotic indicator is satisfactory for migrants of different molecular volumes, a real doubt arises about the advisability of applying so-called correction factors unless absolutely necessary in order to interpret the results. In the experiments described here, the *change* in the mobility was explored as a function of the temperature. Since the effect of temperature on electroosmotic movement appears to parallel that on electromigration mobility, the application of an electroosmotic correction would simply shift the lines, in Fig. 1 and 2, bodily, by a small amount but would not alter the slope or the nature of the equation expressing the effect of temperature on mobilities.

*Summary.* The effect of temperature on velocity of electromigration through paper stabilized electrolytes, of a variety of substances, including bromphenol blue, bovine plasma albumin, human serum albumin and globulin fractions and human serum lipoproteins, was investigated. The ionographic apparatus employed utilized horizontal paper strips in a water saturated atmosphere. The runs were made at temperatures from 4°C to 25°C and under constant conditions of buffer, ionic strength, pH and potential gradient. For all substances studied, the relationship between the mobility and temperature can be represented by the equation:

$$\log \text{ (mobility)} = m \cdot 1/T + B$$

where T is °Kelvin, B is a constant and m is the slope of the line obtained when the logarithm of the mobility is plotted against the reciprocal of the absolute temperature.

The authors are grateful to Alvin Dubin, Director, Dept. of Biochemistry, Hektoen Institute of Medical Research, Cook County Hospital, Chicago, for making available to them the pooled human sera.

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Received November 19, 1956. P.S.E.B.M., 1957, v94.

# Effect of Carbon Tetrachloride Injury on Plasma and Liver Vitamin $B_{12}$ Levels. (22990)

Richard S. Yamamoto, Kunio Okuda and Bacon F. Chow
*Department of Biochemistry, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md.*

Serum vit. $B_{12}$ level of patients with certain liver diseases is increased(1,1a). In the very early stage of viral hepatitis there is an abrupt decrease of vit. $B_{12}$ binding capacity in serum. Since liver is a main site of $B_{12}$ storage it seemed of interest to investigate the effect of liver damage by carbon tetrachloride upon $B_{12}$ serum levels, and on $B_{12}$ absorption.

*Methods.* (1) *Administration of carbon tetrachloride.* To produce liver damage in rats, one ml of a solution of carbon tetrachloride dissolved in olive oil was injected subcutaneously to adult male animals of McCollum strain weighing approximately 400 g. The carbon tetrachloride in the one ml olive oil solution was such that each rat received 0.035 to 0.10 ml of carbon tetrachloride per 100 g body weight. (2) *Microbiological determinations of total and alkali-unstable vitamin $B_{12}$ activity in serum or plasma and liver.* Blood was obtained by cardiac puncture under light ether anesthesia. Twenty-five thousandths, 0.05 and 0.075 ml of serum or plasma separated in the usual manner was added directly to 5 ml of Skeggs' media(2). Growth of *Lactobacillus leichmannii* No. 4797 was estimated titrimetrically, after 64 hours incubation and was taken as a measure of total vit. $B_{12}$ activity. To determine vit. $B_{12}$ activity in liver, the organ was weighed and homogenized with one part liver and 19 parts water. An aliquot of the homogenate was mixed with acetate buffer, heated in boiling water bath, diluted to appropriate concentrations, and then added to test media for assay. Since vit. $B_{12}$, unlike other substances with growth promoting activities, is unstable toward alkali(3), liver or serum extracts prepared by heating specimens in acetate buffer at pH = 4.5 and subsequent removal of proteins by centrifugation were heated at 100° for 30 minutes at 0.20 N NaOH concentration. Alkaline solutions were subsequently neutralized and assayed for vit. $B_{12}$ activity as described above. (3) *Determination of radioactivity of vit. $B_{12}$ in urine and feces.* Adult rats were given, either orally or subcutaneously, radioactive vit. $B_{12}$ labeled with $Co^{60}$, with specific activity of 1100 $\mu$c/mg. The animals were kept in individual metabolism cages. Urine and feces were collected for at least two 24 hour periods. Urine and washings were evaporated to 50 ml in a graduated 100 ml brown bottle. Feces were collected for 4 consecutive days, moistened with small amounts of distilled water and finally homogenized with concentrated sulfuric acid. The homogenate was made up to 50 ml volume. Radioactivity in the bottles containing urine or feces specimens was measured with scintillation counter. (4) *Composition of diets.* All animals were fed a casein diet, or soybean meal diet, both consisting of 4% corn oil, 4% salt mixture IV(4) and vitamin supplement containing all known essential vitamins(5) except vit. $B_{12}$. The casein diet contained 20% crude casein and 72% sucrose, while the soybean diet contained 68% soybean meal and 24% sucrose. The remaining 8% is made up of 4% corn oil and 4% salt mixture IV.

*Results.* Effect of carbon tetrachloride administration on plasma vit. $B_{12}$ activity. In study one, 15 adult rats were divided equally into 3 groups. Two of them received 0.035 and 0.07 ml carbon tetrachloride/100 g body weight, the third group received olive oil alone and, therefore, served as control. All animals were bled at 24 hours and again 7 days after administration of carbon tetrachloride. Plasma specimens were analyzed for vit. $B_{12}$ activity. After the second bleeding, the animals were sacrificed; their livers removed and assayed for vit. $B_{12}$ content. The results in Table I indicate that the plasma vit. $B_{12}$ level was increased markedly

TABLE I. Effect of $CCl_4$ Administration on Plasma and Liver Vit. $B_{12}$ Levels in Rat.

| Dose of $CCl_4$ (ml/100 g body wt) | Plasma $B_{12}$ level (m$\gamma$/ml) 24 hr after $CCl_4$ | 7 days | Liver $B_{12}$ level (m$\gamma$ per g wet wt) 7 days |
|---|---|---|---|
| .00 | .656 ± .024* | .775 ± .025 | 202 ± 57 |
| .035 | .871 ± .082 | .809 ± .031 | |
| .07 | 1.174 ± .105 | .857 ± .025 | 190 ± 26 |

Each group contained 5 rats.
* Avg and stand. error of mean.

upon administration of carbon tetrachloride and the increase was greater the higher the dose of carbon tetrachloride given. The increase was observable within 24 hrs, but the levels returned to the control values by the seventh day, at which time the $B_{12}$ levels in livers of both control and treated groups were essentially the same. To ascertain whether the increase in vit. $B_{12}$ activity in plasma was due to the vitamin itself or other substances with vit. $B_{12}$-like activities, a second study was conducted in which the alkali stable fraction was also measured. Three groups of rats (Table II) were bled 24 hours after administration of carbon tetrachloride. The results again indicate elevation of vit. $B_{12}$ serum level after carbon tetrachloride administration. The amount of alkali stable fraction in serum remained the same after this treatment. The elevated total activity was destroyed with NaOH.

Effect of previous dietary history and plasma vit. $B_{12}$ elevation. Increase in the plasma vit. $B_{12}$ level is probably due to its release from storage organs. It is, therefore, of interest to compare the effect of carbon tetrachloride administration on plasma $B_{12}$ levels of rats fed $B_{12}$-containing and vit. $B_{12}$-deficient diets. To this end, 2 groups of weanling rats were placed on casein and on soybean diets, respectively. The protein contents of both diets were approximately 20%. After 3 months of feeding on these diets, the $B_{12}$ contents in livers of the 2 groups of rats were assayed and found to be 230 ± 46 and 122 ± 31 m$\mu$g/g wet liver for casein and soybean fed rats, respectively. The remaining animals were subdivided into 2 groups. One subgroup received carbon tetrachloride and the other received olive oil alone. Twenty-four hours after injection, the animals were bled and the plasma specimens were assayed for vit. $B_{12}$ activity. Our results demonstrate a marked increase in plasma level of vit. $B_{12}$ in the group fed the casein diet from 0.75 m$\mu$g/ml for untreated group to 0.99 ± 0.073 m$\mu$g/ml of the treated group. No significant change was observed in the group receiving the soybean diet; the $B_{12}$ serum levels were 0.50 m$\mu$g/ml for both treated and untreated groups. The low vit. $B_{12}$ content in the vegetable protein was reflected in low plasma vit. $B_{12}$ level.

TABLE II. Effect of $CCl_4$ Administration on Serum Total Vit. $B_{12}$ and Alkali Stable $B_{12}$ Levels (24 Hr after $CCl_4$ Admin.).

| Dose of $CCl_4$ (ml/100 g wt) | No. of rats | Total serum $B_{12}$ level (m$\gamma$/ml) | Alkali-stable $B_{12}$ activity (m$\gamma$/ml) |
|---|---|---|---|
| .00 | 5 | .401 ± .008 | .210 ± .016 |
| .05 | 5 | .659 ± .059 | .235 ± .008 |
| .10 | 6 | .737 ± .051 | .236 ± .012 |

To test the hypothesis that increase in plasma vit. $B_{12}$ is due to release of vit. $B_{12}$ from the storage organs or tissues, 10 rats (experiment A) were given subcutaneously 20 m$\mu$g of radioactive vit. $B_{12}$ for 3 consecutive days. Urine collection was started 48 hours after the last injection of the radiovitamin. A small amount of radioactivity was found in the urine. These animals were then divided into 2 groups of 5 each. One group received carbon tetrachloride in olive oil and the other group received olive oil alone. Urine and feces collections were continued for the measurement of radioactivity. The results shown in Table III indicate that urinary but not fecal excretion of radioactivity increased markedly in animals treated with carbon tetrachloride.

In another experiment (B), the test animals were divided into 2 groups, one group received carbon tetrachloride and the other received olive oil alone. Twenty-four hours later, both groups of animals were given 20 m$\mu$g of radioactive vit. $B_{12}$ subcutaneously. Urine and feces were collected for 4 days for radioactivity measurement. The results again demonstrate that the injection of carbon tetrachloride resulted in greater excretion

TABLE III. Excretion of Radioactive Vit. $B_{12}$ (Each Group Contained 5 rats).

| Exp. | Dose of $CCl_4$ (ml/100 g wt) | Route of $B_{12}$* admin. | Time of $B_{12}$* admin. | Urinary excretion of radioactivity (mγ) 0-24 hr | 24-48 | 48-96 | Total, 4 day | Fecal excretion (mγ) 4 day |
|---|---|---|---|---|---|---|---|---|
| A | .00 | Subcut. | Prior to $CCl_4$ inj. | .47 ± .05 | .41 ± .037 | .41 ± .049 | 1.30 ± .089 | 6.60 ± .15 |
| | .05 | | | .90 ± .038 | .57 ± .049 | .71 ± .053 | 2.29 ± .107 | 6.25 ± .25 |
| B | .00 | " | After $CCl_4$ inj. | 1.04 ± .054 | .24 | .36 | 1.64 ± .07 | 2.51 ± .15 |
| | .05 | | | 1.46 ± .078 | .27 | .23 | 1.98 ± .17 | 1.94 ± .15 |
| C | .00 | Oral | After $CCl_4$ inj. | .16 ± .04 | .24 | .46 | .86 ± .06 | 19.5 ± 1.8 |
| | .15 | | | .38 ± .03 | .26 | .44 | 1.08 ± .13 | 24.8 ± 1.2 |

of radioactive vit. $B_{12}$ in urine and slightly less excretion in feces.

The effect of carbon tetrachloride on the absorption of orally administered $B_{12}$ was also studied. Ten adult rats (experiment C) were divided into 2 groups of 5 each. One group was treated with carbon tetrachloride and the other, with olive oil. Twenty-four hours afterwards, 50 mμg of radioactive vit. $B_{12}$ in one ml was given by a stomach tube to every animal. The radioactivity in urine and fecal specimens was measured. The results demonstrate a larger amount of radioactivity in urine specimens of treated rats. A slight increase in fecal excretion was likewise observed.

*Discussion.* Vit. $B_{12}$ serum level may be useful in determining the reserve of this vitamin in subjects with different diseases. It can be categorically stated that in clinically recognizable vit. $B_{12}$ deficiency, as in patients with either Addisonian pernicious anemia or anemia due to fish tapeworm infestation or to poor absorption following total gastrectomy or to the dietary lack of vit. $B_{12}$ (vegans), the vit. $B_{12}$ serum level is always extremely low. On the other hand, there are also instances where vit. $B_{12}$ deficiency is believed to exist clinically and yet the plasma level of vit. $B_{12}$ is elevated above normal. For example, diabetics with retinopathy(6) possess higher vit. $B_{12}$ levels than clinically healthy non-diabetics. Elevation of vit. $B_{12}$ level in sera of subjects with leukemia has also been reported(7,8). Our studies suggest that the interpretation of vit. $B_{12}$ levels in serum must take into account the existence of an agent (chemical or physiological) which may temporarily release vit. $B_{12}$ from storage organs. On the other hand, a low level of vit. $B_{12}$ may be considered as a measure of poor reserve. A high level, particularly those with abnormally elevated values, need not indicate sufficiency of vit. $B_{12}$.

The results of our experiments lead us to believe that administration of carbon tetrachloride causes liver injury with consequent release of vit. $B_{12}$, although our data do not rule out the possibility that other organs for $B_{12}$ storage, *e.g.*, kidneys might have been

damaged in a similar manner. $B_{12}$ enters the blood, thus resulting in elevation of vit. $B_{12}$ and in a concomitant increase in urinary excretion. This injury provoked with the amounts of carbon tetrachloride used is apparently only temporary. The elevation of vit. $B_{12}$ activity in serum is most likely due to an increase in vit. $B_{12}$ *per se,* rather than alkali stable factors such as desoxyribosides.

*Summary.* (1) Administration of carbon tetrachloride increased the serum level of vit. $B_{12}$ of rats fed a casein diet. No similar increase was observed in rats previously offered a low vit. $B_{12}$ diet. (2) Administration of carbon tetrachloride likewise increased the urinary excretion of parenterally administered vit. $B_{12}$ or of absorbed vit. $B_{12}$ following oral administration. (3) Physiological significances of these findings are discussed.

The authors acknowledge with thanks a Grant-in-Aid from Merck & Co., Inc., and U.S.P.H.S. Grant.

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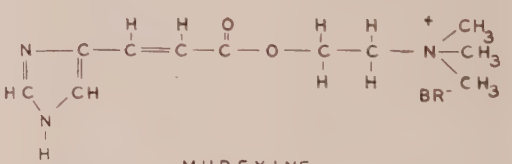
Received November 20, 1956. P.S.E.B.M., 1957, v94.

## Interrelationship of Murexine, Dihydromurexine and Human Cholinesterases.* (22991)

FRANCIS F. FOLDES, ERVIN G. ERDÖS, NORA BAART AND SYDNEY P. SHANOR
*Mercy Hospital and Department of Surgery, University of Pittsburgh School of Medicine, Pa.*

It has been shown by Vincent and Julien (1) and later by Erspamer(2) that extracts from the hypobranchial glands of *Murex trunculus* contain several pharmacologically active agents. One of these, murexine (Mur) was identified as ($\beta$-(4-Imidazolyl)acrylyl) choline or the choline ester of urocanic acid (3).† The compound was first synthesized by Pasini *et al.*(4). Mur has a nicotinic effect on the autonomic ganglia and blocks myoneural transmission in laboratory animals(5) and man(6). Dihydromurexine (DhMur), ($\beta$-(4-Imidazolyl)propionyl) choline, the reduced derivative of Mur, (Fig. 1) is about 4 times as potent as Mur, at the autonomic ganglia and at the neuromuscular



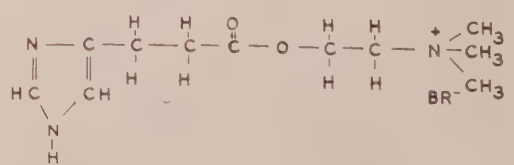




FIG. 1. Structural formulae of murexine and dihydromurexine.

* This investigation was supported in part by Research Grant from N.I.H., U.S.P.H.S.

† Murexine, dihydromurexine and urocanic acid (impurity less than 1%) were synthesized by Hoffmann-LaRoche, Nutley, N. J. and supplied by Dr. Leo A. Pirk.

junction; its duration of action, however, is considerably shorter than that of Mur. DhMur is less toxic in mice than Mur. In clinical trials(6), Mur proved to be a short acting depolarizing neuromuscular blocking agent. It was half as potent as succinylcholine in man.

Because of their pharmacological effect on physiological mechanisms associated with acetylcholine (ACh) metabolism, it seemed worthwhile to investigate the interrelationship of Mur and DhMur with human cholinesterases.

*Material and methods.* Stock solutions of Mur bromide, DhMur bromide and urocanic acid were made up with distilled water. The source of plasma cholinesterase was Cholase.‡ Cholase is the solution of a human plasma cholinesterase concentrate prepared similarly to Harvard fraction IV-6-3. The cholinesterase activity of 1 ml of Cholase with ACh substrate corresponds to 160 ml fresh, pooled, heparinized human plasma. "True" cholinesterase was prepared by hemolyzing washed heparinized human red blood cells. The hydrolysis of Mur by plasma cholinesterase was studied with an ultraviolet spectrophotometric method and with Ammon's manometric method(7). The hydrolysis of DhMur could only be assayed with the manometric technic since its u.v. absorption spectrum is unsuitable for analysis. The absorption spectra of Mur and DhMur are presented in Fig. 2. Since Mur is hydrolyzed by Cholase to urocanic acid and choline, the absorption spectrum of urocanic acid is also shown in Fig. 2. The rate of hydrolysis of Mur was followed by measuring decrease of absorption at a wave length of 317 m$\mu$ in Beckman model D.U. spectrophotometer. The system consisted of 5 x $10^{-5}$ M/lit. Mur and Cholase in 1:10,000 dilution in a pH 7.4 phosphate buffer. The quartz absorption cell had a 10 mm light path. Temperature of the absorption chamber was kept constant at 37°C. The blank contained the same dilution of Cholase in phosphate buffer. The enzyme and substrate were mixed at zero time and readings were taken at 30 second intervals until completion of hydrolysis. Hydrolysis of Mur and DhMur was also investigated with Ammon's technic(7), at 37°C and pH 7.4 using a bicarbonate-carbonate buffer system. The initial concentration of Mur in the experiments was 2.2 x $10^{-2}$ M/lit. and Cholase was diluted 1:800. In the DhMur experiments, the initial substrate concentration was 2.2 x $10^{-2}$ M/lit. but Cholase was diluted 1:8000. Readings were taken at 5 minute intervals for 35 minutes. Michaelis constants ($K_m$) were determined according to Lineweaver and Burk (8) by plotting S/V against S. The $K_m$ of Mur was determined in the spectrophotometer and that of DhMur in the manometric apparatus. The anticholinesterase effect of compounds was studied with the manometric technic. Cholase or red cell cholinesterase was the source of enzyme; ACh, benzoylcholine (BeCh) and butyrylcholine (BuCh) were used as substrates. The $I_{50}$ values were

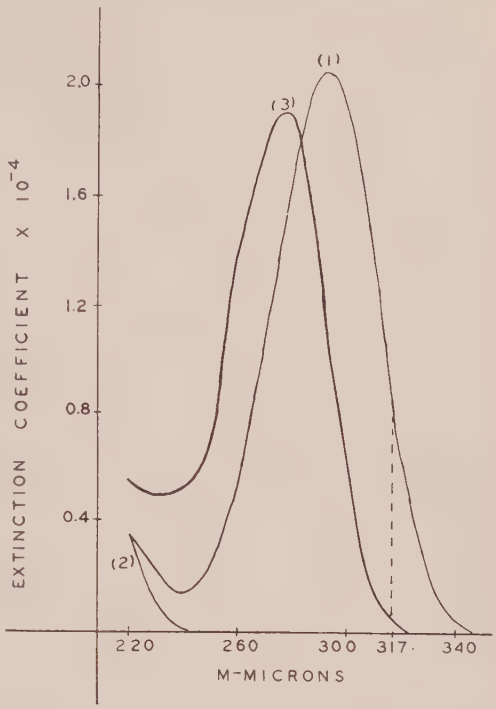



FIG. 2. U.V. absorption spectra of murexine (1), dihydromurexine (2) and urocanic acid (3) at pH 7.4.

‡ Cholase used in this study was kindly supplied by Dr. Edwin B. McLean of Cutter Laboratories, Berkeley, Calif.

TABLE I. Hydrolysis of Murexine and Dihydromurexine by Concentrated Human Plasma Cholinesterase (Cholase).

| Substrate | Hydrolysis rate in Cholase (mol./ml Cholase/30 min.) Manometric method | Spectrophotometric method | $K_m$ (M)/l |
|---|---|---|---|
| Murexine | $1.3 \times 10^{-3}$ | $1.3 \times 10^{-3}$ | $1.1 \times 10^{-5}$ |
| Dihydromurexine | $2.3 \times 10^{-2}$ | | $1.3 \times 10^{-4}$ |

calculated according to Wilson's(9) formula,

$$V/V_i = 1 + \frac{K_m}{K_i}\left[\frac{I}{K_m + S}\right].$$

*Results.* Red cell cholinesterase ("true" cholinesterase) did not hydrolyze either Mur or DhMur. The results obtained with hydrolysis of Mur and DhMur by Cholase are presented in Table I. This table shows that the hydrolysis rates of Mur determined with spectrophotometric and manometric methods were identical. DhMur was hydrolyzed as fast as ACh and 18 times faster than Mur. The spontaneous hydrolysis of Mur and DhMur was very slow under our experimental conditions. In calculating enzymatic hydrolysis rates, corrections were made for alkaline hydrolysis. The $K_m$ of Mur was found to be an order lower than that of DhMur, indicating the higher affinity of Mur to this enzyme. The $K_m$ of ACh ($1.9 \times 10^{-3}$ M/lit) indicates that its affinity to plasma cholinesterase is lower than those of Mur and DhMur. Prostigmine inhibited hydrolysis of Mur and DhMur by Cholase.

Table II demonstrates the inhibitory effect of Mur, DhMur and urocanic acid on human cholinesterases.

Mur inhibited the hydrolysis of ACh and BuCh by Cholase and that of ACh by red cell cholinesterase. Mur had almost no effect on hydrolysis of BeCh. DhMur did not inhibit Cholase in the Warburg experiments. Both Mur and DhMur are rather poor inhibitors of cholinesterases. For comparison the $I_{50}$ of prostigmine, with ACh as substrate, is in the order of $10^{-8}$ M in Cholase and $10^{-7}$ M in red cell cholinesterase.

*Discussion.* Our results indicate that both Mur and DhMur are hydrolyzed by human plasma cholinesterase, but they are unaffected by "true" human red cell cholinesterase. Earlier, Erspamer(2,10) stated that Mur is not broken down by cholinesterases. This discrepancy between the findings of Erspamer and ours is probably due to the fact that the enzyme preparations (calf and rabbit blood and various tissue extracts) used by him contained mostly "true" cholinesterase.

It is of interest that there was an 18 fold difference between hydrolysis rates of Mur and DhMur. The faster hydrolysis rate of DhMur would explain its shorter duration of action and its lower toxicity in mice. The reason for the faster hydrolysis rate of DhMur cannot be explained as yet. It is possible that it is caused by a greater flexibility of the saturated side chain.

The failure of Mur to inhibit hydrolysis of BeCh by Cholase, as contrasted to other substrates, may be due to the low $K_m$ (high affinity) of this substrate to human plasma cholinesterase.

The lack of inhibitory effect of DhMur against Cholase is probably due to its own rapid hydrolysis by this enzyme which causes its fast disappearance from the system.

Urocanic acid, the hydrolysis product of Mur, is also an intermediary breakdown product of histidine metabolism(11). It is, therefore, conceivable that Mur, the choline ester of urocanic ester, might normally occur

TABLE II. Inhibitory Effect of Murexine, Dihydromurexine and Urocanic Acid.

| Substrate | Enzyme | $I_{50}$ (M)/liter Murexine | Dihydromurexine | Urocanic acid |
|---|---|---|---|---|
| Acetylcholine | Cholase | $8.2 \times 10^{-4}$ | No inhibition | Negligible |
| | RChE* | $3.6 \times 10^{-3}$ | $2.4 \times 10^{-3}$ | No inhibition |
| Benzoylcholine | Cholase | Negligible | No inhibition | Negligible |
| Butyrylcholine | " | $4.4 \times 10^{-3}$ | No inhibition | No inhibition |

* Red cell cholinesterase.

in mammals. Indeed, Kewitz(12) found traces of a choline ester of urocanic acid, probably Mur, in extracts of cervical ganglia of cattle and horse.

*Summary.* 1. Murexine (Mur) and dihydromurexine (DhMur) are both hydrolyzed by human plasma cholinesterase. The hydrolysis rate of DhMur is as fast as that of acetylcholine (ACh) and is 18 times faster than that of Mur. 2. Neither Mur nor DhMur are hydrolyzed by human red cell cholinesterase. 3. Both Mur and DhMur inhibit the hydrolysis of ACh by red cell cholinesterase. Mur also inhibits plasma cholinesterase.

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Received December 10, 1956. P.S.E.B.M., 1957, v94.

## Determination of Serum and Tissue Cholesterol. (22992)

ROY G. HERRMANN. (Introduced by K. K. Chen)
*The Lilly Research Laboratories, Indianapolis, Ind.*

It was found by Swinnen(1) and by us that the direct ferric chloride method of MacIntyre(2) yielded high values for total cholesterol of the serum. Furthermore, this procedure was obviously not directly applicable to tissues. By combining this method with the extraction procedure of Abell(3), with minor modifications, a satisfactory method was obtained that eliminated the false high values but retained the desirable color stability and sensitivity.

*Methods.* 1. Alcoholic KOH; 6 ml of a 30% w/w KOH solution are added to 94 ml of redistilled absolute ethyl alcohol and well mixed. A white precipitate may form. This reagent should be prepared just before use. 2. Petroleum ether; 60-70°C boiling range and redistilled. 3. Glacial acetic acid; reagent grade glacial acetic acid is refluxed (2) over chromium trioxide for 3 to 4 hours and then redistilled. Start application of heat cautiously and with constant stirring until mixture is close to boiling point, then remove stirrer and install the reflux condensor. This precaution should be followed to prevent a possible violent exothermic reaction. 4. Color reagent; to about 150 ml of reagent grade sulfuric acid add 10 ml of 10% $FeCl_3 \cdot 6H_2O$ in redistilled glacial acetic acid and mix well, then dilute to 1 liter with concentrated sulfuric acid. 5. Standard cholesterol solution; 20 mg of cholesterol/100 ml of glacial acetic acid. *Tissue preparation*: To known weight of tissue, 100 to 200 mg, add 0.5 ml of 10% tetraethylammonium hydroxide and heat in boiling water bath, with occasional mixing, until solution is attained (about 45 min). Allow solution to cool to room temperature, then dilute to exactly 1 ml with distilled water. The use of tetraethylammonium hydroxide makes possible a further analysis of the sample for alkali earth metals. An aliquot of this is used for determination of cholesterol.

*Procedure.* Place 0.1 ml of serum (0.2 ml in the case of tissue) or less, as required by

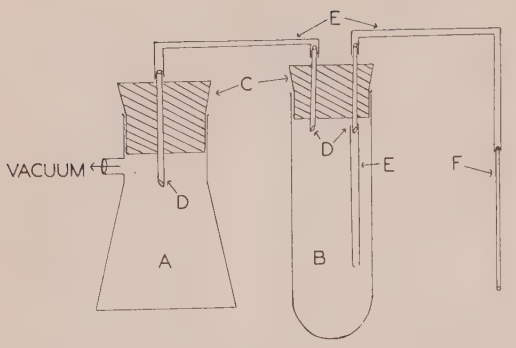


FIG. 1. Aspiration apparatus. A, suction flask; B, colorimeter tube; C, rubber stoppers; D, No. 22 gauge hypodermic needles with slip-on hub removed; E, polyethylene tubing PE50; F, glass capillary which is placed into the petroleum layer to be transferred.

the amount of cholesterol present, into a glass tube (culture tube 14 x 100 mm outside dimensions) and add 1 ml of alcoholic KOH. Mix well and incubate tubes in water bath at 37-40°C for 1 hour. Remove tubes from water bath and, when cool, introduce 2 ml of petroleum ether into each tube via hypodermic syringe with sufficient force to provide mixing. One ml of distilled water is then added and the cholesterol extracted by up and down movement of footed glass rod for 2 minutes. This is most easily accomplished by extracting 10 to 12 tubes simultaneously by a manifold which consists of wooden block holding 10 to 12 footed glass rods spaced so as to fit into tubes in the test tube rack. The emulsion which forms is broken by low speed centrifugation and the top petroleum ether layer containing extracted cholesterol is quantitatively transferred into clean colorimeter tube. This is most readily done by aspiration, (Fig. 1). Petroleum ether is evaporated at room temperature to dryness by gentle stream of air directed into tube from a manifold, (Fig. 2). The stream of


FIG. 2. Air manifold. A, rubber suction tubing; B, cork; C, No. 18 gauge hypodermic needles with slip-on hub removed. A short piece of polyethylene tubing PE160 may be attached if desired.

air is turned off as soon as dryness is attained. To the dry residue add 6 ml of glacial acetic acid and then forcefully introduce 4 ml of color reagent directly into the glacial acetic acid by a 10 ml hypodermic syringe. This allows for instant mixing and avoids uneven heating of solution. Immediately after the color reagent has been added a footed glass rod is employed to insure thorough mixing. The tubes are allowed to cool to room temperature and the color density is then read at 570 mμ. The color is stable for at least 2 hours. A standard curve is prepared by adding from 0.1 to 1.0 ml of standard cholesterol solution to the colorimeter tubes and enough of glacial acetic acid to make total volume 6 ml. Color is developed as stated above.

*Results.* The present method was compared with the direct ferric chloride method of MacIntyre(2) on 32 normal and hypercholesterolemic rabbit sera ranging from 49 to 495 mg% of cholesterol. The values obtained averaged 21% lower (15-25% extremes) than those obtained with the direct ferric chloride procedure. The present procedure was also compared with that of Abell (3) on 8 rabbit serum samples. The results are shown in Table I with good agreement.

TABLE I. Comparison of Rabbit Serum Cholesterol Concentrations, in 8 Samples.

| Serum cholesterol, mg % | |
|---|---|
| Present method | Method of Abell(3) |
| 68 | 65 |
| 36 | 38 |
| 40 | 41 |
| 124 | 125 |
| 101 | 105 |
| 45 | 43 |
| 45 | 42 |
| 44 | 46 |

Color of solution obtained from digestion of most tissues is reddish-brown, which on vigorous agitation turns to a greenish color. This color change had no effect on subsequent cholesterol determination. Furthermore, the tissue digest can be stored in freezer for at least 2 weeks without any effect on cholesterol value. A 500 mg liver sample from a rat on a hypercholesterolemic diet was digested as

TABLE II. Recovery of Cholesterol Added to Tissues.

| Tissue | Rat No. | Cholesterol added (μg) | Cholesterol recovered (μg) |
|---|---|---|---|
| Liver | 1 | 250 | 242 |
| | 2 | 250 | 238 |
| | 1 | 1500 | 1494 |
| | 2 | 1500 | 1418 |
| Serum | 1 | 250 | 229 |
| | 2 | 250 | 255 |
| | 1 | 1500 | 1419 |
| | 2 | 1500 | 1530 |

described. After very gentle mixing of the digest, an aliquot was removed for analysis; another was taken from the reheated digest after vigorous stirring which produced the greenish color; and a third aliquot was taken for analysis after 14 days storage in the freezer. The cholesterol concentrations were 263, 264 and 262 mg/100 g of liver, respectively. Recovery of added cholesterol from serum and liver was also studied. Results presented in Table II indicate that good recoveries are obtained.

*Summary.* A simple and sensitive method for determination of serum and tissue total cholesterol is described.

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Received December 11, 1956. P.S.E.B.M., 1957, v94.

## Glycolytic Intermediates of Human Platelets: Their Separation and Identification.* (22993)

EDMUND W. CAMPBELL, WALTER J. SMALL, EDITH LOPILATO, AND WILLIAM DAMESHEK

*New England Center Hospital, and Department of Medicine, Tufts University School of Medicine, Boston*

Studies of human platelets have indicated the presence of an active carbohydrate metabolism(1,2) with a respiratory quotient of 0.96. Direct investigation of this metabolic system has demonstrated the presence of carbohydrate intermediates, following which partial separation and identification of the esters was obtained. Addition of radioactive phosphorous to the platelets prior to separation led to knowledge of the esters of highest specific activity, and thus to their place in the cycle. Important differences were found between fresh normal platelets, normal stored platelets, and platelets obtained from patients with hematologic disease.

*Materials and methods.* (a) Preparation of Platelets. Blood was obtained using silicone coated surfaces with one-tenth volume of 1% EDTA as the anticoagulant. Platelet rich plasma was carefully separated by differential centrifugation at 4°C. The volume of platelet rich plasma was standardized at 100 ml, the platelet count adjusted to 275,000/mm$^3$ and the pH maintained at 7.4 with .001M phosphate buffer. One millicurie of radioactive phosphorous was added and the platelet rich plasma incubated for 2 hours at 23°C. Platelets were separated by centrifugation at 1500 PM for 15′ and then washed twice with 100 ml of normal saline at 4°C. (b) Extraction of glycolytic intermediates from the washed platelet mass was obtained through modification of a method previously used in the study of erythrocytes (Fig. 1 and 2)(3,4). The platelets were ground after adding 25 ml of 4°C, 7.5% trichloracetic acid, the extract separated by centrifugation at 4°C, and the residue extracted with 25 ml of 4°C, 5% trichloracetic acid. The com-

* This work was supported by Atomic Energy Com. Contract.

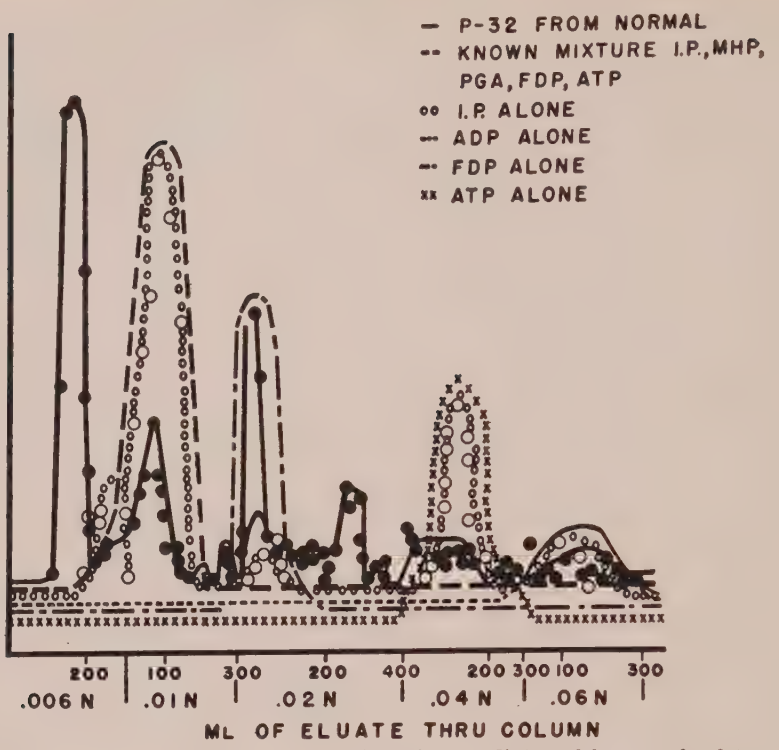


FIG. 1 and 2. Studies on carbohydrate intermediates of human platelets.

bined extracts were neutralized with concentrated ammonium hydroxide, 5 ml of saturated barium chloride was added, and ethonal solution added to final concentration of 80%. Care was taken to minimize hydrolysis by rapid neutralization and low temperatures while the esters were in solution. A minimum time of 2 hours at 4°C was allowed for precipitation of barium salts of the esters. The salts were separated by centrifugation and reconverted to esters by a batch process using hydrogen cycle Dowex-50 which was separated by filtration. The solution of esters was neutralized with ammonium hydroxide and an excess of 1 ml was added. The final volume was adjusted to 10 ml with distilled water. 1.5 ml was retained for $P_{32}$ assay and

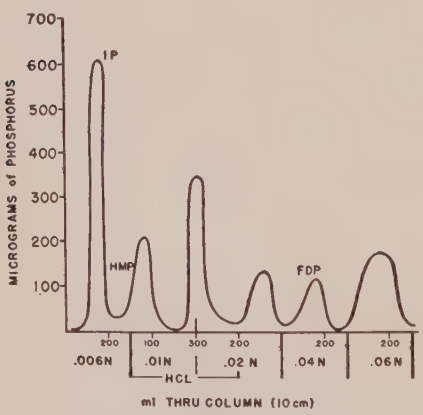


FIG. 3. Phosphate partition of normal human platelets.

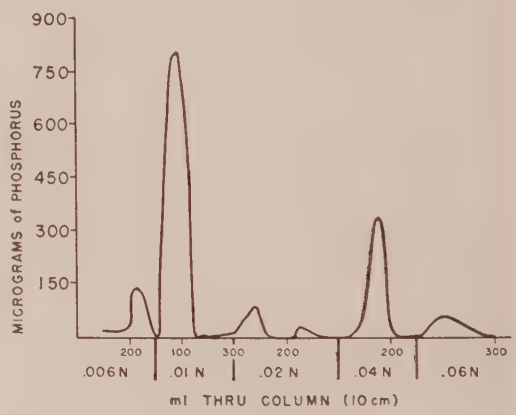


FIG. 4. Phosphate partition of known compounds.

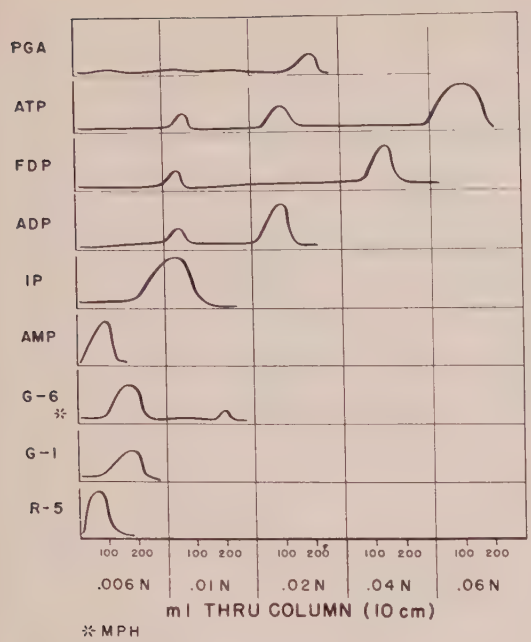


FIG. 5. Composite of data on known compounds by HCl method.

phosphorous analyses, and 8.5 ml was absorbed on a column 1.11 $cm^2$ x 10 cm of chloride cycle Dowex-1 anion exchange resin. Chloride was removed by repeated washing with distilled water and the esters were eluted serially with increasing concentrations of hydrochloric acid. $P_{32}$ was assayed by direct G-M count using a 1.5 $mg/cm^2$ end window tube in conjunction with atomic scaler; phosphorous was determined by the method of Fiske-Subbarow(4). $P_{32}$ activity was corrected for decay to day zero in each experiment and specific activities (S.A.) then calculated.

*Results.*† In an initial experiment using normal human platelets (Fig. 3) consecutive 10 ml samples were collected and an aliquot from each assayed for $P_{32}$. If $P_{32}$ was detected an aliquot was analyzed for phosphorous. Close correlation occurred as would be expected. On the basis of this experiment, a solution containing a mixture of known esters was absorbed and eluted by the same technic (Fig. 4). Subsequently, individual esters of the known mixture were absorbed and the sites of eluation were identified and were consistent with results of Bartlett and Savage (3). A composite of these data was compiled (Fig. 5).

† The following abbreviations have been used: R-5 (ribose 5-phosphate), MHP (mixed hexose phosphates), I.P. (inorganic phosphorous), A.D.P. (adenosine diphosphate), A.T.P. (adenosine triphosphate), A.M.P. (adenosine monophosphate), P.G.A. (phosphoglyceric acid), and F.D.P. (fructose diphosphate).

TABLE I. Partition Data from Normal Platelets.*

| Fraction | 1 | | 2 | | 3 | | 4 | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\mu g$ P/$10^{10}$ | S.A. | $\mu g$ P/$10^{10}$ | S.A. | $\mu g$ P/$10^{10}$ | S.A. | $\mu g$ P/$10^{10}$ | S.A. | $\mu g$ P/$10^{10}$ | S.A. |
| .006†(0-100)‡ AMP or R-5 | | | | | 5.45 | 580 | 8.65 | 1805 | 11.6 | 1865 |
| .006 (100-200) MHP | 19.8 | 670 | 17.2 | 400 | 4.7 | 27.2 | | | 5.2 | 254 |
| .006 (200) + .01 (0-100) IP | 31.2 | 247 | 17.2 | 283 | 8.45 | 51.6 | 6.4 | 73.3 | 7.2 | 341 |
| .02 (0-100) ADP | 11.0 | 345 | 11.0 | 296 | 3.52 | 175 | 7.05 | 651 | 6.4 | 190 |
| .02 (150-200) PGA | | | | | 5.45 | 21.6 | 4.3 | 570 | 4.8 | 51.4 |
| .04 (100-200) FDP | 3.3 | 135 | 7.7 | 76.3 | 4.3 | 13.1 | 3.84 | 24.7 | 4.0 | 111 |
| .06 (0-150) ATP | 4.6 | 156 | 6.1 | 119 | 5.13 | 9.0 | 5.45 | 3.3 | 23.7 | 50.4 |
| Total platelets ext./$10^{10}$ | 125 | | 89 | | 54.6 | | 54.6 | | 61.1 | |
| % platelets recovered | 65 | | — | | 72.5 | | 72 | | 100 | |

Fraction abbreviations—see (†) of text.
* Values in $\mu g/10^{10}$ platelets. † Molarity of HCl. ‡ Vol of sample in ml.

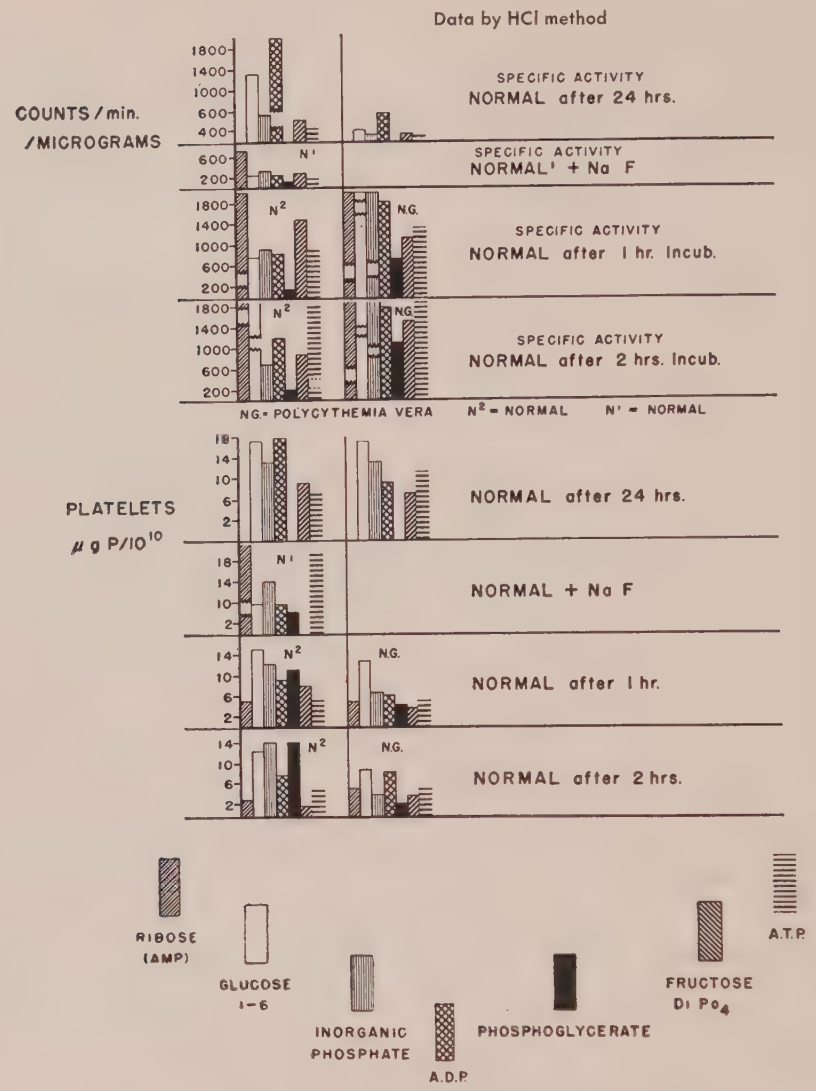


FIG. 6. Glycolytic intermediates of normal human platelets.

Extraction of normal platelets from fresh blood (Table I) yielded 55 $\mu$g of phosphorous/$10^{10}$ platelets with a recovery of 70% of the total esters. The relation of specific activities of the identifiable fractions was R-5, MHP, ADP, IP, PGA, FDP and ATP in descending order with some variation. Numerical differences occurred in all experiments but these values were obviously less important than their relative differences and order of specific activity. In all experiments, HMP and ADP were of nearly equal activity.

Study of freshly collected, normal platelets stored for 24 hours at 4°C revealed some differences (Fig. 6). From initial data the total amount of phosphate esters of glycolytic intermediates extracted was decreased with recovery of 75-85% of the esters. The decrease in total amount of esters extracted suggested a leakage of phosphorous from platelets into the medium during storage. Notably, the quantity of ADP differed with a marked increase in the specific activity as compared with minimal difference in MHP indicating a qualitative change. The PGA fraction was also decreased.

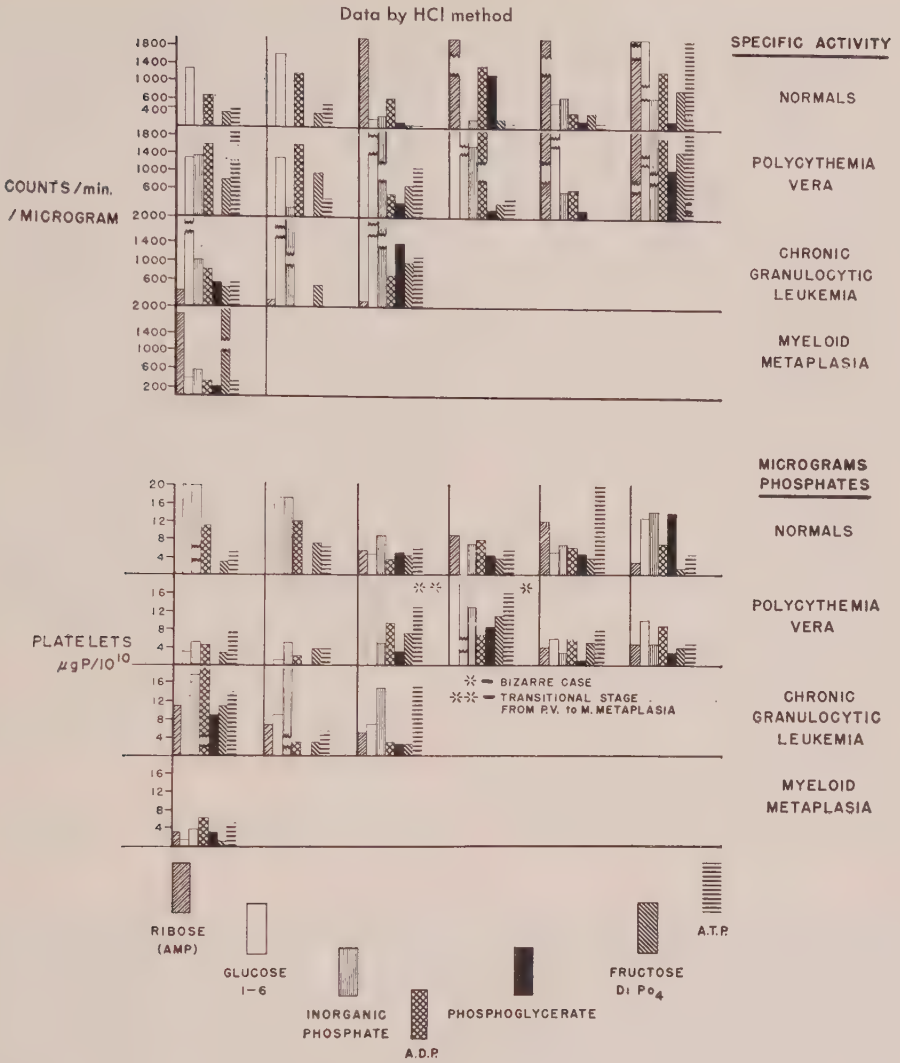


FIG. 7. Comparative study of the glycolytic intermediates of platelets in hematologic diseases.

Similar study of platelets obtained from individuals having polycythemia vera, chronic granulocytic leukemia and myeloid metaplasia (Fig. 7) revealed striking differences in platelets in these different diseases as compared with normals. In polycythemia vera 40 $\mu$g of Phosphorous/$10^{10}$ platelets was obtained as compared with 119 mg in the platelets of chronic granulocytic leukemia.

A decrease in quantity of I.P. was obtained from platelets of 6 individuals with polycythemia while a significant increase in those from chronic granulocytic leukemia was observed. A decrease of similar magnitude was detected in the ADP, PGA, FDP, and ATP fractions. The specific activities of the esters of polycythemic platelets indicated a reversal from normal in R-5, and in MHP with an increase in ATP although there was considerable variation in different individuals. In platelets from 5 cases of chronic granulocytic leukemia the specific activity of R-5 was very markedly decreased. Several fractions were separated which have not been identified but had higher specific activities than the identified esters. Study of platelets of 3 cases of

myeloid metaplasia have revealed very bizarre individual variations suggesting marked variations in this syndrome.

*Discussion.* Direct study of carbohydrate metabolism of human platelets through fractionation of glycolytic intermediates revealed important differences as between fresh normal platelets, stored normal platelets, and the platelets of individuals with certain hematologic disorders (Fig. 7). (Individual variations have occurred and may be explained by variation in differences in hematologic syndromes.)

Comparison of carbohydrate intermediates from fresh normal and stored normal platelets revealed that storage resulted in a decrease in total amount of extractable esters. This suggested a leakage of phosphorous from platelets to plasma. The simultaneous increase in ADP with its marked increase in specific activity as compared with MHP suggested that stored platelets had undergone a qualitative metabolic change. Changes in specific activity of the various fractions indicated a qualitative change in this cycle. The striking qualitative and quantitative variations from normal values in platelets of chronic granulocytic leukemia suggested the possibility of an abnormal carbohydrate metabolism. Of particular interest was the increase of inorganic phosphorous and the presence of several unidentified fractions.

*Summary.* Presence of active degree of carbohydrate metabolism and identification and separation of some intermediates of this system has been directly demonstrated in human platelets. The use of radioactive phosphorous to determine specific activity of some fractions has yielded important additional data. Preliminary studies have indicated quantitative and qualitative alterations in carbohydrate metabolism during storage of normal platelets and particularly in platelets obtained from certain hematologic disorders, notably chronic granulocytic leukemia.

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Received December 18, 1956. P.S.E.B.M., 1957, v94.

## Dye-Dilution Curves from Left Heart and Aorta for Localization of Left-to-Right Shunts and Detection of Valvular Insufficiency. (22994)

EUGENE BRAUNWALD, HERBERT L. TANENBAUM AND ANDREW G. MORROW
(Introduced by Robert W. Berliner)
*National Heart Institute, Bethesda, Md.*

The precise localization of left-to-right shunts whether intra- or extracardiac, may sometimes be difficult or even impossible with conventional diagnostic technics. A similar problem is presented in the detection of mitral or aortic valvular insufficiency. Dilution curves recorded from a peripheral artery following injection of indicator into a peripheral vein, the right heart, or pulmonary artery are helpful in establishing the presence of a left-to-right shunt(1). They are of relatively little value, however, in determining its precise location(2).

The technics for catheterization of the left side of heart(3,4,5) and of the central aorta (6) have made possible the injection of an indicator into the left atrium, left ventricle, and various sites in the aorta. Dilution curves obtained from these injection sites have been recorded in patients with various types of left-to-right shunts or valvular insufficiency.

*Methods.* Left heart catheterization was usually performed by the transbronchial route(5). In several patients the catheter was passed into the left heart retrograde from

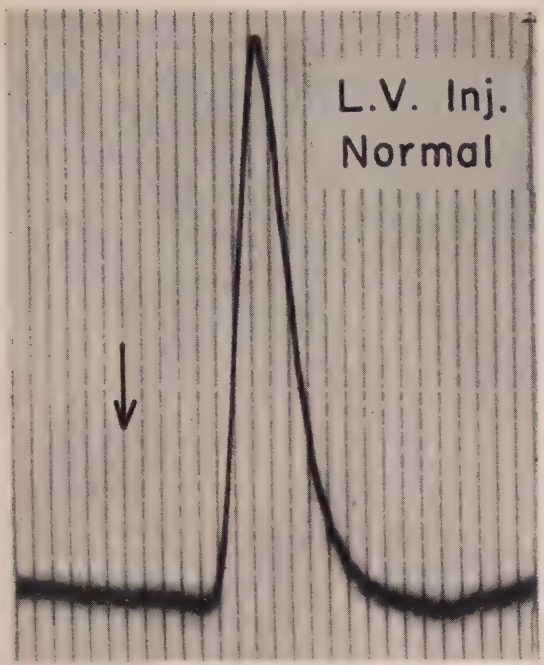


FIG. 1. Dye dilution curve following inj. of Indigo Carmine into ascending aorta of patient with aortic stenosis but without either aortic regurgitation or a left-to-right shunt. Arrow indicates instant of inj. Time lines equal 1 sec.

a peripheral artery. In patients with interatrial communications the catheter was passed via the saphenous vein, through the defect and into the left heart. Central aortic catheterization was performed through a No. 12 thin-walled needle introduced percutaneously into the femoral artery, or via the surgically exposed right ulnar artery. The indicator used was either Evans Blue or Indigo Carmine(7). A cuvette densitometer* was employed in conjunction with a photographic cathode-ray recorder for continuous recording of dye concentrations from femoral artery.

*Results.* In the absence of any left-to-right shunt, or of valvular regurgitation on left side of heart, the rapid injection of an indicator into the left atrium, left ventricle or thoracic aorta yielded a primary curve with a sharp rapid ascent and a slightly slower descent (Fig. 1). When the injection was made proximal to the site of a left-to-right shunt, that fraction of the indicator which passed through the pulmonary circulation distinctly interrupted the smooth, rapidly descending limb of the primary curve with an abrupt decline in rate of fall in concentration. However, when the indicator was injected distal to site of shunt a normal curve was obtained (Fig. 2). Thus, by selectively injecting the indicator into various sites the location of the left-to-right shunt could be

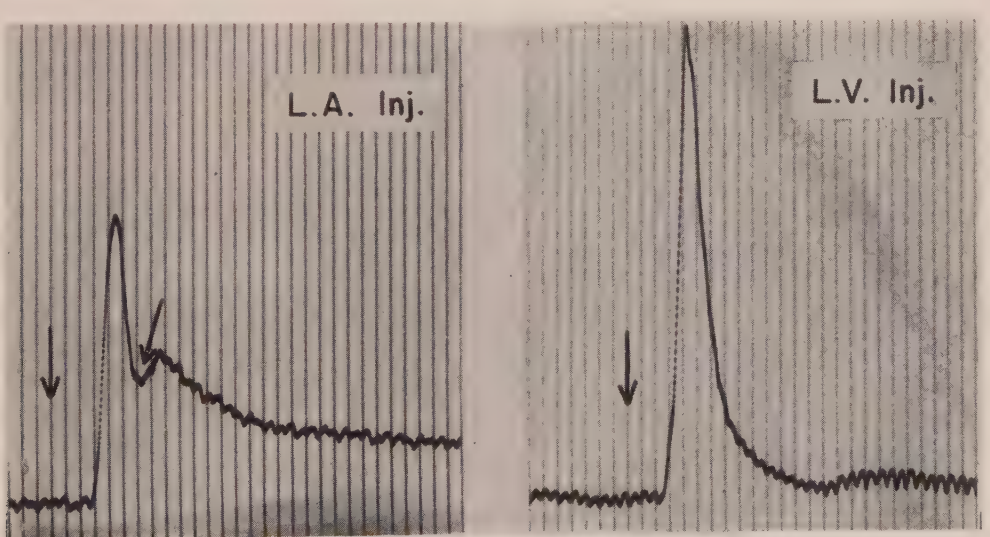


FIG. 2. Dye dilution curves in patient with uncomplicated interatrial septal defect. Curve on the left resulted from left atrial inj. and shows presence of a left-to-right shunt (oblique arrow). Curve on the right was into the left ventricle and is normal.

* Colson Corp., Elyria, O.

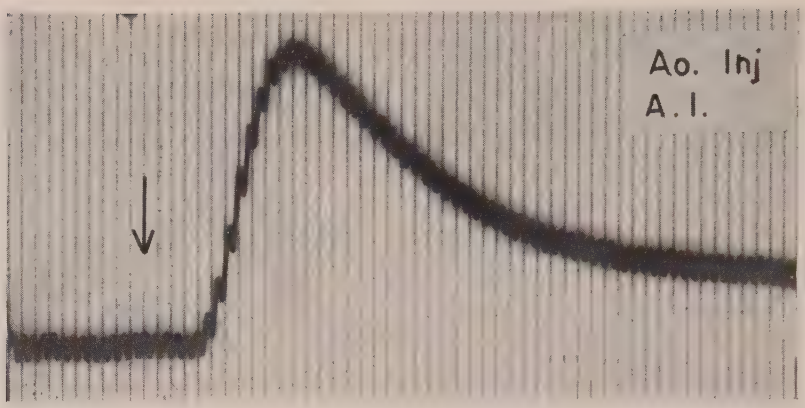


FIG. 3. Dye dilution curve following inj. into the ascending aorta of patient with aortic regurgitation.

precisely determined.

When the injection was made immediately distal to an insufficient valve, a portion of indicator washed back and forth. This resulted in a dilution curve with rapid ascent but a distinctly prolonged, generally smooth uninterrupted descent (Fig. 3).

Thirty-three patients have been studied to date. No complications have resulted from the left heart or arterial catheterizations. The technic has proved particularly useful in distinguishing between: 1) interventricular septal defect and aortic septal defect; 2) aortic septal defect and patent ductus arteriosus; 3) aortic valvular disease and ruptured aneurysm of sinus of Valsalva; and, 4) aortic insufficiency and pulmonic insufficiency. Another application has been in the determination of the presence or absence of an interventricular septal defect or of mitral insufficiency in patients with known interatrial septal defects, *i.e.*, establishment or exclusion of the diagnosis of a common atrioventricular canal. In patients with anomalous pulmonary venous drainage the presence or absence of an interatrial septal defect has been determined.

*Summary.* A technic is described which makes possible the precise localization of left-to-right intra- or extracardiac shunts and the detection of aortic and mitral valvular insufficiency. An indicator dye is rapidly injected into chambers of the left heart or aorta and a dilution curve is recorded from a peripheral artery. The method has been applied in 33 patients and has proven of diagnostic value.

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Received December 26, 1956. P.S.E.B.M., 1957, v94.

# Study of Folic Acid and Vit. $B_{12}$ in Blood and Urine During Normal Pregnancy.* (22995)

HERMAN BAKER, RUTH ERDBERG, INEZ PASHER, AND HARRY SOBOTKA
*Departments of Chemistry, Obstetrics and Gynecology, Mt. Sinai Hospital, New York City*

This report deals with blood and urine levels of cyanocobalamin (vit. $B_{12}$) and folic acid (PGA) simultaneously determined in the same subjects during pregnancy, not complicated by hemorrhage and abortion.. Recent investigations have shown vit. $B_{12}$ level in serum to be low during pregnancy(1,2). In the latter communication, mean levels of vit. $B_{12}$ in serum before delivery were as low as those reported in cases of pernicious anemia (3). Since the functions of vit. $B_{12}$ and PGA are interrelated, the present study was undertaken in an attempt to correlate their involvement during pregnancy.

*Methods.* Methods for determination of vit. $B_{12}$ in serum and urine have been described(4,5). All assays for vit. $B_{12}$ were made in parallel using *Euglena gracilis* (Z strain) and *Ochromonas malhamensis;* a comparison which permits elimination of responses to congeners of the vitamin(4). PGA in urine was estimated with a thermophilic bacillus(6).† A modification of this method for assay of blood was devised, as suggested by earlier work.‡ Blood is diluted 1:5 with citrate buffer at pH 6.1, to which 0.05% ascorbic acid has been added. After incubating the blood-buffer mixture for 2 hours, the proteins can be completely removed after a 10 minute autoclaving period by centrifugation; the supernatant is then diluted again with medium 1:2-1:100 (final dilutions 1:10-1:500). Recovery experiments using this technic yielded satisfactory results for a range from 0.3-100 mμg/ml. Patients were selected from the pre-natal clinic of the hospital. The cases that developed complications during pregnancy were excluded. The patients received vitamin preparations free of PGA and vit. $B_{12}$ to avoid interference with the assay. Ordinary diets do not perceptibly disturb PGA levels(7), however vit. $B_{12}$ levels might be altered; blood and urine were obtained in the morning; patients were not placed on controlled diet. Several blood and urine determinations were carried out on the same patients whenever possible during duration of pregnancy. A total of 47 patients were studied.

TABLE I. $B_{12}$ and PGA Content of Blood and Urine during Normal Pregnancy.

| Quartile | $B_{12}$ (μμg/ml) Blood | $B_{12}$ (μμg/ml) Urine | PGA (mμg/ml) Blood | PGA (mμg/ml) Urine |
|---|---|---|---|---|
| 1st | 80–108 | 0–19 | 0–10 | 0–10 |
| 2nd | 108–193 | 19–55 | 10–19 | 10–18 |
| 3rd | 193–360 | 55–95 | 19–57 | 18–72 |
| 4th | >360 | >95 | >57 | >72 |
| Median | 193 | 55 | 19 | 18 |
| No. of patients | 51 | 54 | 54 | 54 |

*Results.* The occasional high values in these determinations tend to distort the picture, if it were represented by average values. We have therefore given the medians, rather than the means, in the following presentation.

*$B_{12}$ in urine and blood.* The vit. $B_{12}$ content in urine and blood is given in Table I. Fifty-four specimens were included in the urine study, and 51 in blood studies. The median of urinary $B_{12}$ levels was 55 μμg/ml and of blood levels 193 μμg/ml. Moreover, 35 instances of $B_{12}$ in urine were crowded in the group below 60 μμg/ml; of these, 14 in the group below 25 μμg/ml. In blood values, 17, or one-third of the total, were in the group below 125 μμg/ml.

The PGA content of urine and blood was determined in 54 specimens; results are rendered in Table I. The median of the PGA level in urine and blood was 19 mμg/ml. In both cases the distribution curve shows a

* Aided by grants from U. S. Public Health Service and Am. Cyanamid Co., Lederle Laboratories.

† The basal medium for this assay is under revision and will be reported separately. The medium as used now, contains 2% glycerol, inst. of 2% sucrose, because some sucrose samples contain a PGA-like substance which contaminates the assay medium and results in high blanks.

‡ Reported in *Fed. Proc.,* 1954, p. 311.

much slower decrease towards higher values than in normal non-pregnant subjects.

*Discussion.* The study indicates that PGA increases during pregnancy in blood and urine. Judging from previous reports on PGA levels in blood and urine of normal cases(3, 8,9), the median value of 19 m$\mu$g/ml is from 2-8 times as high as normal. Since the renal threshold for PGA is low, it is not surprising that the median urinary PGA values are also increased above the normal values reported.

Fetal development poses a problem of cellular and tissue synthesis for the pregnant woman. The need for facilitation of all mechanisms involving such synthesis is pressing; PGA and $B_{12}$ are known to be involved (10). Any inhibition of PGA can lead to fetal death or developmental abnormalities. This effect has been shown in chick embryos (11). Because pregnancy places abnormal demands on maternal metabolism, the available stores of these vitamins are brought into circulation to continue this state and to further fetal development.

PGA is also a factor in hormonal response; estrogen activity in birds and mammals has been inhibited by PGA deficiency(12). Aminopterin, a PGA antimetabolite, can terminate pregnancy by inhibiting progesterone activity. Since estrogen and progesterone are vital hormones in pregnancy, it is likely that PGA is involved in pregnancy by enhancing hormonal activity or overcoming any inhibition. Ovarian hormones fail to maintain pregnancy in PGA deficient animals(13); it is thus reasonable to assume that in this deficient state hormonal inhibitors are operative. The finding of high levels of PGA during pregnancy, previously reported(14), and also observed by us, supports the assumption that PGA is needed to insure proper hormonal activity by antagonizing inhibitors.

Vit. $B_{12}$ levels in this study were low. Median urine values were 2-5 times lower than normal(3,15), and blood values up to 4 times lower. The low serum $B_{12}$ observed, as pregnancy progressed, suggests that the fetus may attract maternal $B_{12}$ across the placenta, thereby causing a depletion of this vitamin in the maternal circulation. Recent results bear out this premise(2,16). A study of placental transfer of $B_{12}$, as compared with PGA, is now under way in our laboratories.

It is clear from our results that pregnancy exacts overwhelming demands on PGA and $B_{12}$ stores. High maternal stores of these vitamins during pregnancy should alleviate some of the stress, which fetal development places upon maternal metabolism, and could prevent intrauterine injury(17).

*Summary.* A comparison of PGA and vit. $B_{12}$ levels in blood and urine during pregnancy shows that levels of PGA are high, and those of $B_{12}$ are low. Possible explanations for these findings are discussed.

We are indebted to Dr. Alan F. Guttmacher, Director of Department of Obstetrics and Gynecology of Mt. Sinai Hospital, and to Dr. Lazar Margulies for generous cooperation.

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Received January 7, 1957. P.S.E.B.M., 1957, v94.

## Comparison of Some Suggested Anticariogenic Compounds in the Rat.* (22996)

JOSEPH C. MUHLER
*Indiana University, Bloomington, Ind.*

This report concerns an evaluation of 4 substances which have been suggested for reducing human dental caries by means of topical procedures. These include stannous fluoride(1,2), sodium fluoride(3), sodium oxalate (4), and sodium N-palmitoyl sarcosinate(5).

*Materials and methods.* A total of 280 weanling Sprague-Dawley strain rats were divided according to initial body weight into 7 groups. Each group was further subdivided equally according to sex. Groups 1, 2, and 3 received sodium fluoride, stannous fluoride,† or sodium fluorostannite‡ respectively, at a fluoride concentration of 30 parts/million. Group 4 received sodium oxalate at an oxalate concentration of 30 parts/million. Group 5 received 1% solution of sodium N-palmitoyl sarcosinate. Group 6 received a solution of sodium N-palmitoyl sarcosinate and sodium fluoride, the concentrations of which were identical with those which were used in Groups 1 and 5 above. Group 7 received distilled water and served as control. In all instances the compounds were put in drinking water, which the animals received *ad libitum*. All solutions were made fresh each morning, and any solutions remaining in the drinking tubes from previous day were discarded. No attempt was made to regulate amount of solution the animals received. However, frequent estimates were made concerning amount of water which an entire group drank during a 24-hour period. These data initially indicated that the 2 sarcosinate groups and the stannous fluoride groups consumed somewhat less solution, but after about 30 days these differences were not pronounced. The animals all received the same stock corn cariogenic diet§ and were housed in pairs in raised-screen cages in air-conditioned room. The duration of the study was 140 days, after which the animals were sacrificed by ether and the heads removed for dental caries evaluation as previously described(6).

*Results.* A summary of the pertinent data (Table I) indicates that the 3 fluorides were the only substances tested which numerically decreased the mean number of dental cavities, when compared to the control. However, of the 3 fluorides only stannous fluoride and sodium fluorostannite were significantly effective (probability of 0.05). When sodium fluoride was added to the sarcosinate solution at the same concentration as in Group 1, no effect on dental caries was observed. The reason for the apparent difference between the 2 sodium fluoride groups, with and without sarcosinate, is not known. Sodium oxalate was without effect, while 2 groups receiving sodium N-palmitoyl sarcosinate had an apparent but not significant increase in incidence of dental caries.

In both groups receiving the sarcosinate solution there was less weight gain during the

* This study was supported in part by grant from Procter and Gamble, Ivorydale, O.

† Obtained from Metal and Thermit Co., Rahway, N. J.

‡ Synthesized in Department of Chemistry at Indiana University.

§ Composition of diet was as follows: yellow corn grits, 52.7%; ground yellow whole corn, 11.3%; powdered whole milk, 30%; alfalfa, 4.8%; iodized sodium chloride, 1%; and irradiated yeast, .2%.

TABLE I. Comparison of Ability of Potential Anticariogenic Substances to Reduce Incidence of Dental Caries in the Rat.

| Group | No. of animals | Dental caries experience: Mean No. of lesions | Molars affected | % change* |
|---|---|---|---|---|
| Sodium fluoride | 32 | 8.0 ± 1.4† | 3.7 | −17 |
| Stannous fluoride | 30 | 7.1 ± 1.1 | 3.5 | −26 |
| Sodium fluorostannite | 32 | 6.8 ± 1.0 | 3.5 | −29 |
| Sodium oxalate | 29 | 9.8 ± 1.8 | 4.7 | + 7 |
| Sodium N-palmitoyl sarcosinate | 29 | 10.7 ± 2.0 | 5.5 | +15 |
| *Idem* + sodium fluoride | 34 | 10.3 ± 1.6 | 4.8 | +12 |
| Control | 30 | 9.6 ± 1.5 | 4.5 | — |

* Based upon comparison between mean No. of lesions in control and various groups.
† Stand. dev.

experiment than in the control group. In the group receiving sarcosinate solution, the final weight of males was 284 g, of females 206 g, as compared to 345 g and 226 g for males and females in the control group. Similarly, the male group receiving sarcosinate and sodium fluoride weighed 288 g and the females 198 g. These were the only groups which did not weigh as much as, or more than, the controls.

Table II compares our data, concerning the effect of these substances on enamel solubility, dental caries in rats, and human caries experience. It is seen from this Table that sodium fluoride was less effective in reducing both powdered enamel solubility and rat dental caries, but was effective in reducing human dental caries. Similarly, sodium oxalate was ineffective in reducing powdered enamel solubility as well as rat dental caries, while sodium N-palmitoyl sarcosinate was only moderately effective in reducing powdered enamel solubility, and ineffective in reducing rat dental caries. It is hoped that these materials will be carefully tested with respect to their ability to reduce caries in humans, in order that the presence or absence of correlations among the several methods can eventually be established.

TABLE II. Comparison between Effects of 5 Different Compounds on Powdered Enamel Solubility and on Rat Dental Caries. Correlation with human dental caries is indicated where justified on the basis of published clinical studies.

| | % reduction in powdered enamel solubility* | % reduction in dental caries in rats | Effect in human clinical studies |
|---|---|---|---|
| Sodium fluoride | 44 | 17 | positive(1,3) |
| Stannous " | 86 | 26 | " (1,7) |
| Sodium fluorostannite | 85 | 29 | not available |
| Sodium N-palmitoyl sarcosinate | 20 | 0 | *Idem* |
| Sodium oxalate | 1 | 0 | " |

* Previously published(8) and used here for comparative purposes only.

As judged by these experiments, the animal studies seem to predict the general usefulness of fluorides in reducing dental caries by topical procedures. If these correlations are correct they would also suggest the usefulness of sodium fluorostannite, since it also produced significantly less dental caries, but, to be valid for humans this finding on rats must be confirmed by the demonstration of reduction in human dental caries.

Much additional work is needed before anything definite can be said regarding these correlations. However, at present there appears to be a good correlation between results obtained on enamel solubility and rat dental caries.

*Summary.* The effectiveness of sodium fluoride, stannous fluoride, sodium fluorostannite, sodium oxalate, and sodium N-palmitoyl sarcosinate was compared in their ability to reduce experimental dental caries in the rat. Only fluorides resulted in less dental caries, while stannous fluoride and sodium fluorostannite were significantly different from controls. Sodium fluoride and sarcosinate fed together had no effect on the caries rate.

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Received January 7, 1957. P.S.E.B.M., 1957, v94.

## Regulation by Anterior Pituitary Hormones of Nucleic Acids in Dependent Endocrine Glands.*† (22997)

SILVIO FIALA, EDITH E. SPROUL, AND ANNA E. FIALA
(Introduced by A. Gellhorn)
*Department of Pathology, Columbia College of Physicians and Surgeons, and Francis Delafield Hospital, New York City*

The anterior pituitary gland regulates by means of tropic hormones such as TSH (Thyreotropin), FSH (Follicle stimulating hormone) and ACTH (Corticotropin) the activities of thyroid glands, ovarian follicles and adrenal cortex. Stimulation may be manifest in various ways in different organs but increase in weight and enlargement of target tissue always occur after repeated hormone administration, while involution follows hypophysectomy. It seemed plausible that an identical reaction in each of these glands may be provoked by its corresponding hormone, while the unique physiologic response of the gland may be an expression of specific qualities of the respective target cell. Previously (1) we described a rapid increase of cytoplasmic, microsome-bound RNA accompanied or followed by increase in total mass of cytoplasmic elements (mitochondria and microsomes). The increase in ratio RNA/protein nitrogen (basophilic quotient) of the microsome fraction was especially conspicuous and the change from value 1.1 to 1.8 after some 200-300 units of ACTH occurred with few accompanying cell divisions. The RNA, protein and DNA content of isolated nuclei increased later, so that the initial action of this hormone affected the cytoplasm, primarily the microsome RNA.

This work has been extended to determine whether analogous effect on cytoplasmic RNA is exerted by 2 other tropic hormones and whether a reverse effect follows hypophysectomy. Finally it seemed pertinent to inquire whether any difference in level of acid soluble nucleotides precedes the induced synthesis of cytoplasmic RNA.

*Materials and methods.* The hormones used were: purified Thyreotropin (TSH) Armour, activity 0.80 USP unit/mg, purified follicle stimulating hormone (FSH) Armour, activity 0.8 Armour unit/mg, and Corticotropin (ACTH) Armour.‡ All were inoculated i.m. into 200 g Wistar albino rats. Control animals were of the same litter, sex, and within 5% of body weight. The hypophysectomized rats§ were used 3-5 weeks after operation. The pooled glands were homogenized in isotonic sucrose. Differential centrifugation and separation of fractions were carried out as described previously(1). RNA and DNA were extracted by Schneider's method(2) from homogenized tissue or separated fractions and their amount determined colorimetrically. Protein nitrogen was determined by Nesslerization from the residuum after nucleic acid extraction. The results are expressed in absolute amounts present in the

* This investigation was supported by grant-in-aid from Am. Cancer Soc. upon recommendation of Com. on Growth of National Research Council.

† This paper presented at Annual Federation Meetings, Apr. 15, 1956.

‡ Purified TSH and FSH were kindly supplied by Dr. S. L. Steelman of the Research Division of Armour Co., Chicago, Ill.

§ Retropharyngeal hypophysectomy was performed by Mrs. F. J. Agate of the College of Physicians and Surgeons, Columbia University.

TABLE I. Effects of TSH, FSH, and ACTH in Hypophysectomized Rats.

| Exp. | | No. glands pooled | Treatment | Wt of tissue (mg) | Total amt of DNA $\times 10^{-6}$ g | No. of cells† $\times 10^{6}$ | Total amt of RNA $\times 10^{-6}$ g | RNA/cell $\times 10^{-12}$ g |
|---|---|---|---|---|---|---|---|---|
| | | | | Thyroids | | | | |
| TSH | C‡ | 4 | 0 | 24 | 166 | 33 | 122 | 3.69 |
| | E | 4 | 5.5 mg, 4 inj., 24 hr | 31 | 173 | 34.6 (+5%) | 227 | 6.56 (+77%) |
| | | | | Ovaries | | | | |
| FSH | C | 2 | 0 | 76 | 515 | 103 | 835 | 8.1 |
| | E | 2 | 4 mg, 2 inj., 12 hr | 102 | 453 | 90.6 | 1009 | 11.1 (+37%) |
| | E | 2 | 6 mg, 3 inj., 24 hr | 120 | 548 | 109 (+6%) | 1190 | 10.9 (+34%) |
| | | | | Adrenals | | | | |
| ACTH | C | 4 | 0 | 33.2 | 239 | 48 | 180 | 3.76 |
| | E | 4 | 200 units, 5 days | 66 | 239 | 48 | 307 | 6.4 (+70%) |
| *ACTH | C | 4 | 0 | 127 | 446 | 89.2 | 748 | 8.39 |
| | E | 4 | 200 units, 5 days | 162 | 602 | 120.0 (+38%) | 1148 | 9.56 (+14%) |

* The data in this row refer to intact (non-hypophysectomized) animals.
† No. of cells was calculated as units of DNA $\times$ 5.0 $\times 10^{-12}$ g.
‡ C = Control; E = Experiment.

tissue and also calculated/DNA unit, $5 \times 10^{-12}$ g. This value represents DNA content of average diploid nucleus of a rat cell(3). The number of haploid cells in the ovary is too small in proportion to diploid cells to invalidate estimates for an average cell. The level of acid soluble nucleotides was estimated from UV-absorption spectra of perchloric acid extracts. Cooled homogenate was mixed with equal volume of ice-cold 10% perchloric acid and immediately centrifuged at 15000 rpm in refrigerated International centrifuge. Clear supernatants were diluted with 5% perchloric acid to 10 or, if necessary, to 20 ml and the UV-spectra measured in a Beckman DU-spectrophotometer. At pH 0.1, ascorbic acid, which is also present in the perchloric acid extract, has an absorption maximum at 244 m$\mu$. This obscured the measurement of acid soluble nucleotides with absorption maxima at 260 m$\mu$ when adrenal glands were extracted. In ovarian and thyroid tissues this interference was slight. Hence the perchloric acid extract of adrenal glands was neutralized with 0.5 M KOH after titrating an aliquot in the presence of phenolphthalein. The precipitated $KClO_4$ was centrifuged off. In the course of this procedure, or shortly afterwards, the ascorbic acid is oxidized to dehydroascorbic acid which does not absorb light in this UV-region.

*Results.* An example of the effects of TSH, FSH and ACTH on RNA content of thyroid gland, ovaries and adrenals in a series of 15 animals is presented in Table I. The change in DNA content and weight of tissue is also indicated. The amount of RNA increased sharply in all instances, whereas, with the exception of the last row containing data for non-hypophysectomized animals, there was practically no increase in amount of DNA in whole tissue with the dosage used. Similar experiments, performed with TSH and FSH respectively, on slightly older animals, showed analogous results.

Differential centrifugation and separation of subcellular fractions from intact rats injected with FSH has shown a striking increase of cytoplasmic RNA and protein nitrogen (Table II). As was true of ACTH(3), FSH also had its most striking effect on the basophilic quotient of the microsome fraction. The mitochondrial fraction was increased in total amount. However it showed only a small relative increase in B.Q. which may be explained by microsome contamination. In

TABLE II. Effects of FSH on Ovaries of Young Intact Rats.

| Treatment | Wt of tissue (mg) | No. of glands pooled | Total amt DNA (mg) | Mitochondria RNA (mg) | Mitochondria Prot. N. (mg) | Mitochondria B.Q. | Large chromidia (microsomes) RNA (mg) | Large chromidia (microsomes) Prot. N. (mg) | Large chromidia (microsomes) B.Q. | Small chromidia and final supernatant RNA (mg) | Small chromidia and final supernatant Prot. N. (mg) | Small chromidia and final supernatant B.Q. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 214 | 4 | 1,570 | 52.8 | 240 | .22 | 384 | 360 | 1.1 | 1,022 | 875 | 1.16 |
| 4 mg FSH in 48 hr | 388 +81% | 4 | 1,873 +19% | 105 +90% | 360 +50% | .29 | 821 +113% | 430 +20% | 1.90 | 1,646 +60% | 2,000 +130% | .82 |

Other experiments, in which differential centrifugation was not performed, gave the same sharp increase (up to 80%) of total RNA as contrasted with slight increase (8-12%) in DNA.

the supernatant there was an increase in RNA but the increase of protein nitrogen exceeded that of RNA so that the B.Q. of this fraction dropped after hormonal stimulation. The total amount of DNA in ovarian tissue in this series of intact rats increased after hormonal administration; nevertheless it was far below the percent increase of cytoplasmic RNA. Analogous results were obtained with TSH and they were similar to those observed previously with ACTH(1).

It seemed of interest to compare the effect of hypophysectomy with this striking increase of cytoplasmic RNA followed by delayed lesser increase in DNA provoked by administration of hormones of the anterior pituitary (Table III). There was not only a reduced quantity of cytoplasmic RNA in all glands after hypophysectomy but also a sharp drop in DNA content, although there is no evidence that the content of DNA/nucleus changed. From Table III we see that the cells became smaller and less numerous; nevertheless depletion of RNA was not only absolute but also relative/unit of DNA. Hypophysectomy then, exerts an effect on RNA which is the reverse of tropic hormonal action.

All 3 hormones induced an increase of cytoplasmic RNA only after a lag period of several hours. This period was preceded by sharp depletion in level of acid soluble substances absorbed at 260 mμ. Following FSH and TSH administration this reaction was seen in the course of 2 hours (Fig. 1). ACTH had the same effect on the adrenal gland (Fig. 2). In this case the drop in level of acid soluble substances absorbing at 260 mμ occurred while the level of ascorbic acid, known to be greatly depleted from the adrenal within a few minutes after ACTH(4), was still at a low point. The level of acid soluble nucleotides remained low long after ascorbic acid returned to normal.

*Discussion.* The data presented demonstrate that all 3 hormones studied have a similar pattern of action in regard to nucleic acid metabolism. Very shortly after administration, they all induce a decrease in level of acid soluble substances, presumably nucleotides, absorbing at 260 mμ, followed by synthesis of cytoplasmic RNA concentrated in microsomes. This effect is accompanied

TABLE III. Effects of Hypophysectomy on Endocrine Glands.

| Tissue | Treatment | Total wt of tissue (mg) | Total amt of DNA $\times 10^{-6}$ g | No. of cells $\times 10^{6}$ | Total amt of RNA $\times 10^{-6}$ g | RNA/cell $\times 10^{-12}$ g |
|---|---|---|---|---|---|---|
| Adrenals | Control | 191 | 680 | 136 | 1056 | 7.7 |
| | Operated | 50 (−74%) | 342 | 68.4 (−50%) | 297 (−72%) | 4.3 (−45%) |
| Ovaries | Control | 168 | 1329 | 266 | 1069 | 4.0 |
| | Operated | 90 (−47%) | 669 | 164 (−39%) | 594 (−45%) | 3.6 |
| Thyroid | Control | 43 | 243 | 48.6 | 324 | 6.6 |
| | Operated | 27 (−37%) | 193 | 38.6 (−20%) | 129 (−61%) | 3.3 (−50%) |

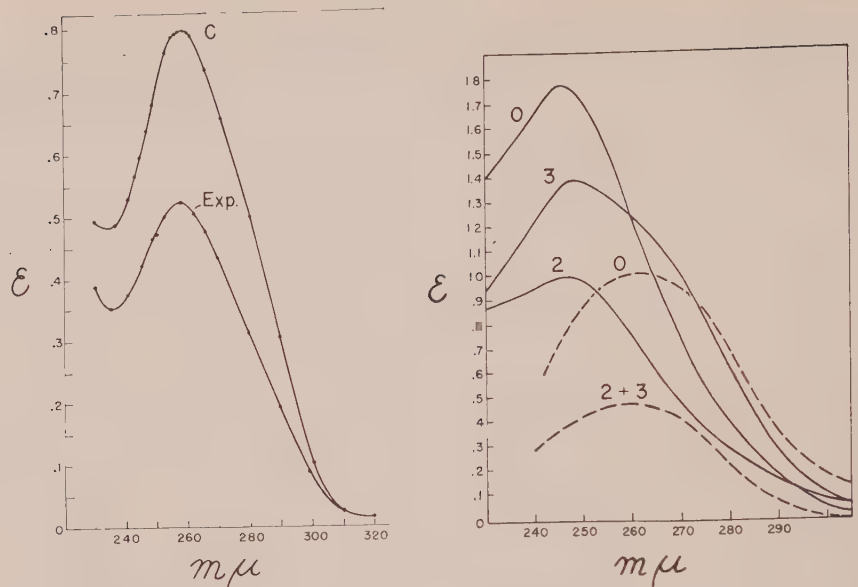


FIG. 1 (left). Effect of FSH on acid soluble nucleotides in rat ovaries. Ovarian extracts (10 ml, 5% perchloric acid) from a control (66 mg of tissue) and experimental rat (tissue wt 68 mg) 2 hr after inj. of 2 mg of purified FSH.

FIG. 2 (right). Perchloric acid extracts from rat adrenals. From a group of 12 male rats (avg wt 150 g) 4 uninj. rats served as controls. Eight were inj. with 25 USP units of ACTH Armour. Four killed 2 hr and four 3½ hr after inj. Perchloric acid extracts made from tissue homogenized in isotonic sucrose. Wt of tissue: controls 133 mg, 139 and 154 mg in 2 hr and 3½ hr experiments. Spectra of acid extracts (full lines) are composite of ascorbic acid (abs. max. at 244 mμ) and of nucleotides (abs. max. 260 mμ). In neutralized extracts absorption of ascorbic acid disappears. Spectra show that 2 and 3 hr after inj., level of nucleotides is on the same low level. At 244 mμ differences between full and broken lines at zero time and 3 hr later are similar, indicating that ascorbic acid returned almost to normal level.

by increase in microsome and mitochondrial protein before there is any change in DNA content. It is therefore independent of cell division. Hypophysectomy acts in an opposite way, lowering the level of RNA/unit of DNA. This fact together with the finding of McShan *et al.*(5) that Luteotropin (LTH) affects the nucleic acid content of stimulated crop gland of the pigeon, indicates that regulation of RNA in target glands is one of the major effects of tropic anterior pituitary hormones.

*Summary.* In endocrine glands such as thyroid, ovary and adrenal cortex the level of cytoplasmic RNA is regulated by the anterior pituitary gland. The amount of RNA increases after hormonal administration and is lowered below the normal level after hypophysectomy. These variations of RNA are to a large extent independent of nuclear DNA which changes to a more limited degree. The hormonally induced synthesis of cytoplasmic RNA is preceded by a sharp drop in level of acid soluble substances, presumably nucleotides, absorbing at 260 mμ.

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Received January 8, 1957. P.S.E.B.M., 1957, v94.

# Changes in Submaxillary Gland Ribonucleic Acid Following Hypophysectomy, Thyroidectomy and Various Hormone Treatments.* (22998)

David Bixler,† Joseph C. Muhler, Richard C. Webster, and William G. Shafer
*Departments of Chemistry and Anatomy and School of Dentistry, University of Indiana, Bloomington.*

Recent studies have indicated that granule content of the tubular portion of rat submaxillary glands is inversely related to incidence of experimental dental caries(1-3). Proteins, or closely related organic substances, seem to be directly involved in this relationship, since proteolytic activity of the rat submaxillary gland appears directly related to dental caries incidence(3). Screebny(4) has proposed that the granular tubule is the site of this proteolytic activity. Since ribonucleic acid is intimately associated with protein metabolism, it seemed desirable to know what changes, if any, in ribonucleic acid content of the granular cells were taking place under those experimental conditions known to alter proteolytic activity and tubule granule content. In addition, this study compares the effects in the submaxillary glands with those observed in the parotid and sublingual rat salivary glands since little information is available on enzymatic aspects of these glands.

*Materials and methods.* This study consisted of 2 experiments. *Series I* was composed of 40 male Sprague-Dawley strain rats hypophysectomized at 45 days of age, and 6 male unoperated Sprague-Dawley strain rats of approximately 55 days of age, which served as controls. Five days following surgery the animals were divided into 4 groups and fed *ad libitum* a special diet of milk, bread, dog food, carrots and oranges. Group 1 received daily injections of 10 $\mu$g of aqueous sodium thyroxine subcutaneously and 200 $\mu$g of testosterone propionate in olive oil intramuscularly. Group 2 received daily intramuscular injections of 2.5 mg of cortisone acetate in aqueous suspension. Group 3 received all 3 hormones daily at the same concentrations described above. Group 4 received no supplements and served as the hypophysectomized control. Group 5 was composed of unoperated animals which served as controls. The animals were sacrificed after 21 days and the salivary glands were removed and fixed in 10% neutral formalin. *Series II* was composed of 30 weanling male rats of the same strain equally divided into 5 groups. Groups 1-4 were intraperitoneally injected, respectively, with 100, 250, 500 and 750 $\mu$c of carrier-free radioactive iodide. All animals were fed a stock laboratory corn diet previously described(5). One hundred days after injections, all animals were sacrificed and major salivary glands and thyroid glands were removed and fixed in 10% neutral formalin. Sections of submaxillary, sublingual and parotid glands were cut at 8 $\mu$ from both series of animals and stained by the methyl green pyronine method described by Kurnick (6). This method is specific enough to yield semi-quantitative data on ribonucleic acid (RNA) distribution and concentration. Additional sections from each gland were incubated in a sterile solution of crystalline ribonuclease‡ (0.25 mg RNase/ml) for 2 hours at 37°C, then stained by methyl green pyronine. Any staining material persisting after the RNase digestion was considered to be material other than RNA.

*Results.* Ribonucleic acid in all 3 major salivary glands was confined to the acinar portion of the gland; no RNA was found in the duct system. For this reason the data obtained from Series I and II, which appear in summary form in Tables I and II respectively, pertain only to the acinar portion of the 3 glands.

Series I. *Submaxillary Gland.* Hypophysectomy markedly depleted the RNA content

* This work was supported in part by Department of Army, under Contract.

† Postdoctoral fellow, N.I.H.

‡ Previously heat-treated at 90°C for 10 min. to destroy any contaminating desoxyribonuclease.

TABLE I. Effect of Hypophysectomy and Hormone Replacement Therapy upon RNA Content of the Major Salivary Glands.

| | Submaxillary gland | | Sublingual gland | | Parotid gland | |
|---|---|---|---|---|---|---|
| | * | † | * | † | * | † |
| Hypo + testosterone & thyroxine | BND | 4 | BN | 4 | BND | 3 |
| " + cortisone | BN | 1 | " | 1 | BN | 1 |
| *Idem* + testosterone & thyroxine | " | 3 | " | 2 | " | 2 |
| Hypophysectomized | " | 0 | B | 0 | " | 1 |
| Control unoperated | " | 2 | " | 2 | " | 2 |

* Denotes location of RNA: B = Basal portion of cell; N = Nucleolar; D = Diffusely spread in cytoplasm.

† Denotes estimated amount of RNA as judged by intensity of staining reaction. 0 = essentially none present; 4 = very heavy conc. of RNA.

TABLE II. Effect of Loss of Thyroid Function on RNA Content of Major Salivary Glands.

| Thyroid histology | | Submaxillary gland | | Sublingual gland | | Parotid gland | |
|---|---|---|---|---|---|---|---|
| | | * | † | * | † | * | † |
| 100 $\mu$c $I^{131}$ | Normal | BN | 4 | BN | 4 | BD | 4 |
| 250 | Some degeneration but still functional | " | 3 | " | 4 | " | 4 |
| 500 | Atrophic | BND | 2 | BND | 2 | " | 3 |
| 750 | " | " | 2 | " | 2 | " | 3 |
| Control | | BN | 4 | BN | 4 | " | 4 |

* Denotes location of RNA: B = Basal location; N = Nucleolar; D = Diffuse.

† Denotes estimated amount of RNA—judged by intensity of staining reaction. 0 = essentially none; 4 = heavy RNA conc.

of acinar cells in the submaxillary gland. The small amount observed was located in basal portions of the cytoplasm and in the nucleolus of each cell. Injection of cortisone (2.5 mg/day) had only a slight effect on RNA content while administration of both testosterone (200 $\mu$g/day) and thyroxine (10 $\mu$g/day) dramatically increased the acinar RNA content. In this instance a small amount of RNA was observed evenly distributed throughout the cytoplasm. Location of acinar RNA following simultaneous administration of all 3 hormones was essentially the same as that observed following only testosterone and thyroxine administration. However, intensity of the RNA stain was not so great as that observed in the group receiving only testosterone and thyroxine. Apparently, cortisone may have an inhibitory effect on cytoplasmic RNA under these conditions.

*Sublingual and Parotid Glands.* Results for these 2 glands were essentially the same as described for the submaxillary gland. That is, hypophysectomy markedly depleted RNA content in both mucous acini and serous acini of the 2 glands. Cortisone had some slight ability to relieve these hypophysectomy-induced changes. However, simultaneous administration of testosterone and thyroxine resulted in an even greater RNA content than in the unoperated animals. When cortisone was used it appeared to exert a damping action on the apparent RNA stimulating ability of testosterone and thyroxine (Table I).

Series II. *Submaxillary Gland.* Ribonucleic acid in control animals was predominately in the basal portion of the cell and in close relationship to the nuclei. Following treatment with 100 $\mu$c of $I^{131}$ concentration and location of RNA remained unchanged, while rats which received 250 $\mu$c of $I^{131}$ seemed to give slightly less intense RNA reaction. Administration of 500 $\mu$c of $I^{131}$ noticeably decreased RNA reaction and, in addition, RNA no longer appeared to be concentrated in basal portion of the cell but distributed diffusely throughout the cell cytoplasm. This same histologic picture was ob-

served in the 750 μc group.

*Sublingual Gland.* The RNA of normal sublingual gland is localized in the basal portion of the mucous acinar cell and lies in close proximity to the basement membrane and nucleus. The groups which had received 100 and 250 μc of $I^{131}$ had essentially the same concentration and distribution of RNA. However, administration of 500 and 750 μc of $I^{131}$ reduced RNA content of cells by approximately one-half and this RNA was not concentrated in any one portion of the cell but diffused throughout the cytoplasm.

*Parotid Gland.* Control animals had parotid glands which showed RNA concentrated in the basal portion of the acinar cell. Administration of radioactive iodine to any group did not change cellular location or concentration of RNA in any of the 4 experimental groups.

Histologic study of thyroid glands from Series II animals revealed that increasing the dose of radioactive iodide resulted in progressive destruction of thyroid tissue. Both control and 100 μc animals receiving $I^{131}$ had identical, normal appearing thyroid glands. Thyroid glands from the 250 μc group showed definite but minimal effects of radiation. Many cells exhibited a frothy appearing colloid while a few had no colloid at all. Only an occasional pycnotic nucleus was observed and the epithelium in the majority of follicles was low cuboidal. Some connective tissue invasion was apparent.

The glands from the 500 and 750 μc groups were essentially identical and will be described together. Only a few follicles remained in these glands and these were atypical and non-functional in appearance. The bulk of the gland in both groups was composed of fibrous connective tissue. These results appear in Table II. The effects of altered thyroid function on salivary gland RNA also appear in Table II.

*Discussion.* The results of these experiments indicate that ribonucleic acid content of major salivary glands is related to normal thyroid and testicular function. This appears to be true since administration of thyroxine and testosterone completely inhibited depletion of RNA following hypophysectomy. Administration of the 2 hormones to hypophysectomized rats resulted in what appeared to be supranormal amounts of RNA in the acinar cells. It is felt that food intake plays a negligible role in explaining the results of this study because of the short duration of the experiment, and since the final body weights were essentially identical, the hypophysectomized group having a mean weight of 103 g and the hypophysectomized group receiving testosterone and thyroxine 110 g.

The thyroid gland appears to have the most prominent effect upon acinar RNA content and localization, since a progressive loss of thyroid tissue was paralleled by progressive loss of RNA and a change in its cellular location. Shafer *et al.* have shown that only thyroxine and testosterone can prevent marked decrease in proteolytic activity of the rat submaxillary gland following hypophysectomy (7). They also found that other hormones such as cortisone, or growth hormone may partially alleviate these changes.

It was noted that RNA of the parotid gland was least affected by either destruction of the thyroid gland with $I^{131}$ or by throxine administration. The submaxillary and sublingual gland RNA, however, demonstrated a pronounced responsiveness to altered thyroid function. More interesting, the tubular portion of the submaxillary gland, which several workers have associated with dental caries experience, contained no RNA. Since it was the acinar portion of the gland which showed alterations in RNA following various hormone treatments, these results would seem to indicate that the study of a dental caries-salivary gland relationship must include both a study of the entire gland as well as a study of each one of the salivary glands.

Baker and Abrams(8) have studied the effect of hypophysectomy and hormone replacement on the weight and histologic appearance of the major salivary glands. They found that of the major salivary glands the parotid gland is most severely affected by hypophysectomy. In addition, thyroxine did not restore the gland weight loss following hypophysectomy. No gland weights were

taken in this study, but, histologically, the submaxillary gland appeared the most drastically affected by hypophysectomy.

*Summary.* 1) A histochemical study of location and concentration of RNA in the major salivary glands was made in animals which had been subjected to various experimental procedures. It was noted that only the acinar portion of the salivary gland exhibited RNA. None was found in the duct portion of any of the 3 major salivary glands. In the hypophysectomized rat RNA is strikingly reduced in the acini of all major salivary glands. Injection of cortisone does not appreciably affect this condition whereas thyroxine and testosterone in combination restore the RNA to normal concentration and location. 2) A progressive loss of thyroid function produced by injecting graded doses of $I^{131}$ resulted in progressive loss of cytoplasmic RNA. This effect was obtained in submaxillary and sublingual glands. The parotid gland RNA was essentially unaffected by thyroidectomy.

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Received January 8, 1957. P.S.E.B.M., 1957, v94.

## Effect of Estrogens on Myocardial Sensitivity to Toxic Effects of Digoxin.* (22999)

EDWARD H. GRINNELL† AND PAUL W. SMITH

*Department of Pharmacology, University of Oklahoma School of Medicine, Oklahoma City.*

In experiments previously reported at the Federation meetings, 1955, it was found that a single dose of 0.15 mg of digoxin/kg body weight, given intravenously to dogs under pentobarbital anesthesia, produced an arrhythmia characterized by complete A-V dissociation and persistent idioventricular tachycardia. The interval between administration of the glycoside and onset of idioventricular rhythm varied from 5 to more than 30 minutes. It was of considerable interest to us that female animals, without exception, exhibited the greatest delay in onset of arrhythmia. This observation suggested that susceptibility to this particular manifestation of digitalis intoxication might be influenced by estrogenic hormones. To investigate this possibility, a new series of experiments was instituted, in which the time interval was measured with the greatest possible accuracy.

That such an influence exists is shown by data presented below, which includes experiments on normal males, normal females in anestrus, normal females in natural estrus, castrate females and castrate females treated with estrogenic drugs.

*Materials and methods.* Forty-four healthy, adult, mongrel dogs of both sexes, weighing 7 to 20 kg, were used. Of the 44, seven were normal males, 4 were normal females in anestrus, 3 were normal females in natural estrus and 30 were castrate females. The castrate females were spayed by technic which included both bilateral oophorectomy

* This research was supported by grants from Council on Pharmacy and Chemistry, Amer. Med. Assn. and Research Com., University of Oklahoma Medical Center.

The digoxin used was given to us by Burroughs Wellcome & Co., (U.S.A.). The estradiol was synthesized in the laboratory of Dr. Max N. Huffman.

† Research Fellow of American Heart Assn.

TABLE I. Influence of Estrogens on Susceptibility to Digoxin Intoxication.

| No. animals | Avg time for onset of arrhythmia (min.) | Range (min.) | Fate |
|---|---|---|---|
| 4 normal ♀, anestrus | 26.0 | 23– 31 | All survived. |
| 7 normal ♂ | 13.6 | 6– 17 | Ventricular fibrillation; 5 dogs died; one treated, survived; one exp. terminated early. |
| 13 castrate ♀, estrogen treated | 31.7 | 23– 45 | All survived. |
| 17 castrate ♀ | 14.3 | 5– 22 | Ventricular fibrillation; all died. |
| 3 normal ♀, natural estrus | 71.6 | 45–120 | All survived. |

and hysterectomy. The castrate animals were divided into 2 groups. Seventeen served as controls and the remaining 13 were treated with one of 3 estrogenic substances injected intramuscularly according to the following dosage schedule: Estrone 2 mg/day; Estradiol 2 mg/day, Diethylstilbestrol 10 mg/day. Experiments on estrogen-treated castrate female dogs were performed when the animals were in peak estrus as proved by vaginal smear technic. Acute experiments were carried out in the same manner on all animals. Food was withheld for 18 to 24 hours prior to experiment. Water was allowed *ad libitum* during the fasting period. The animal was anesthetized with pentobarbital sodium, 30 mg/kg body weight, and connected to a Cathode-Ray Electrocardiograph for continuous observation of electrocardiogram. Lead aVf was routinely employed. After normal electrocardiogram had been observed, digoxin was given intravenously at 0.15 mg/kg of body weight, and time of administration recorded. Time was again recorded when digoxin-induced idioventricular rhythm had become fully established. In the discussion and the Table we refer to this critical endpoint as the "onset" of arrhythmia. Animals in which ventricular fibrillation did not occur during observation period of 3 to 4 hours, are referred to as having survived, whether or not they were killed to terminate the experiment or saved for later use.

*Results.* The results of experiments are summarized in Table I. It can be seen that for 4 normal female dogs in anestrus, the average time required for onset of arrhythmia was 26 minutes, and not one fatality occurred. On the other hand, the average time of onset of arrhythmia in 7 normal males was 13.6 minutes, and 5 of the 7 died of ventricular fibrillation. One experiment was terminated for technical reasons shortly after onset of arrhythmia, and one animal survived after treatment with potassium ethylenediamine tetraacetate according to our previous description (*Federation Proceedings,* 1955).

Untreated castrate females exhibited greatly increased sensitivity to toxic action of digoxin, indicated by average time interval of only 14.3 minutes. All 17 animals in this group died of ventricular fibrillation, the majority within one hour. The castrate female and the normal male are, therefore, almost identical with regard to their relative susceptibility to the toxic action of digoxin on cardiac rhythm.

The average interval of onset of the arrhythmia in 13 estrogen-treated castrate females was 31.7 minutes, which is not significantly different from the average for normal females in anestrus. All 13 of the estrogen-treated castrate females survived. The 3 estrogenic substances used appeared to be equally effective in restoring resistance of the castrate female animal.

Normal female dogs in natural estrus and nonpregnant, are difficult to obtain from our usual source of supply. The 3 animals of this type included in the Table show a resistance to digitalis which cannot be duplicated by estrogenic hormones alone in the castrate animal.

*Summary and conclusion.* The data in our study indicate that estrogenic hormones exert a protective action against the toxic effects of digoxin on the myocardium. Male dogs and castrate female dogs have been

found to be highly sensitive to toxic manifestations of a cardiac glycoside on cardiac rhythm, in comparison with normal females in anestrus, or castrate females treated with estrogenic substance. A high degree of resistance to toxicity of digoxin was exhibited by the 3 normal females in natural estrus which were available.

Received January 14, 1957. P.S.E.B.M., 1957, v94.

## A Hook-like Instrument for Atraumatic Ligation of Minute Vessels.* (23000)

ANNA SIREK (With technical assistance of Y. Hanus)
(Introduced by C. H. Best)
*Banting and Best Department of Medical Research, University of Toronto, Canada.*

The complete interruption of arterial blood flow to liver in rat presents a technical problem because of the wide anatomical variations and the very small size of vessels to be ligated. At least 3 arteries have to be tied. Chase Greene(1) describes the common hepatic artery, the gastroduodenal artery and the hepatic artery proper. Moreover, De Long(2) found a small collateral artery in rats, arising from the esophageal artery and joining the intrahepatic branches of the hepatic artery proper. This artery always increased in size and became readily visible when he ligated the other three.

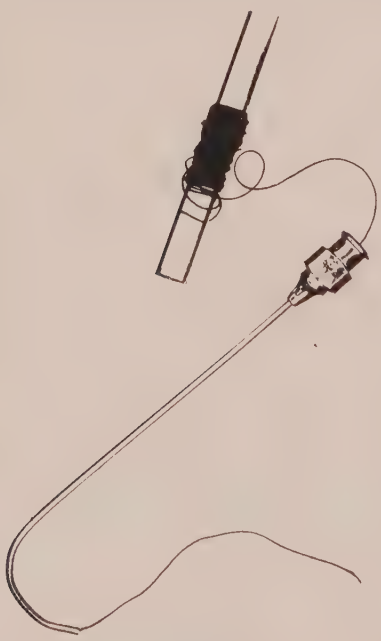

FIG. 1. Crochet hook-like instrument. Description in text.

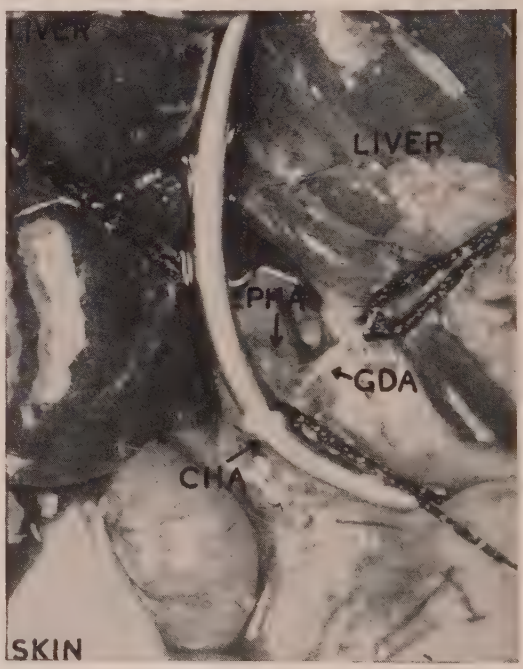


FIG. 2. First step of operation: Liver turned cranially. Gastroduodenal artery (GDA) and hepatic propria artery (PHA) are ligated. Hook passes thread around common hepatic artery (CHA). Note how large the #000 silk seems in comparison to minute size of vessels.

Having these anatomical details in mind, we were faced with the technical problem of ligating all 4 vessels with a minimum of

* The author is indebted to Dr. Benito Lombardi, whose biochemical and histological studies in cirrhotic livers of rats have stimulated this work.

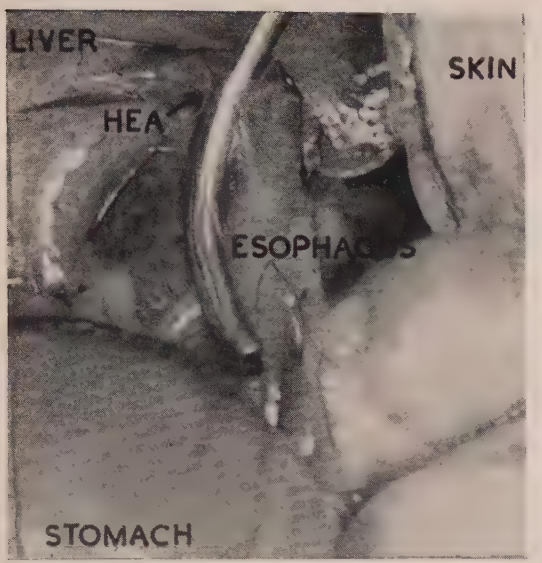


FIG. 3. Second step of operation: Liver turned to right side. Hook passes thread around hepato-esophageal artery (HEA).

trauma and in the shortest possible time.

*Methods.* An 18 gauge-4 inch lumbar-puncture needle bent into a hook proved to be of great help (Fig. 1). The free end of the needle was bent into an arc about 2 cm in diameter and its tip was blunted. The needle was threaded with #000 surgical silk with the free end protruding from the tip and the spool at the other end of the needle. This hook is used in the same way as an aneurysm-hook, but does not need to be rethreaded each time. The needle is passed around the blood vessel, the end of thread is caught and the hook is then removed.

There is no need for any extensive dissection as the hook is simply passed blindly around the vessel and emerges from the surrounding tissues. The entire procedure of ligation takes only a fraction of the time which would be required if a complete dissection had to be carried out and the thread passed around the vessel by a curved haemostat.

Fig. 2 shows the first step of the operation. The liver has been turned cranially. The gastroduodenal artery and the hepatic artery proper have been ligated. Fig. 3 shows the second step of the operation. The liver has been turned to the right. The hook passes the thread around the hepato-esophageal artery.

*Summary.* A hook shaped needle facilitating the ligation of minute vessels in the rat is described. It has been adapted from an 18 gauge needle used originally for lumbar puncture. The instrument has been used with success for complete interruption of the arterial blood supply to the liver of the rat.

The assistance of Mr. C. R. Cowan in making the instrument is gratefully acknowledged.

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Received January 14, 1957. P.S.E.B.M., 1957, v94.

# Presence of Progesterone in Extracts of Ovaries of Laying Hens.* (23001)

D. S. Layne, R. H. Common, W. A. Maw and R. M. Fraps
*Faculty of Agriculture, Macdonald College of McGill University, P.Q., Canada and Agricultural Research Service, U.S.D.A., Beltsville, Md.*

The post-ovulatory history of ovarian follicles in birds differs from that in mammals; and there does not appear to be any structure in the bird analogous to the mammalian corpus luteum. However, extracts of avian blood have been shown to have progestogenic activity(1), as assayed by the Hooker-Forbes (2) test, and various experiments on effects of exogenous progesterone, administered either alone or in conjunction with estrogen, suggest that progesterone, or hormone of equivalent effect, is produced in the laying hen and is important in its reproductive function(3). We have obtained chromatographic and spectrometric evidence for the presence of progesterone in ovarian follicles of laying hens.

*Methods.* Ovaries were removed from 25 laying hens immediately on killing. The large yellow ova were at once removed from their follicles and discarded and the residual ovarian tissue and follicles were disintegrated in methanol-ether (3:1) in a Waring blendor. Extraction of steroid material was carried out as described by Edgar(4). The final extract was chromatographed on filter paper (Whatman No. 1) by use of ligroin—80% methanol system(5). Examination of the paper under ultraviolet hand lamp revealed a prominent absorbing spot at the $R_f$ realized for pure progesterone under similar conditions. The spot reacted positively to 2, 4-dinitrophenylhydrazine. The spot was eluted from the paper with ethanol and the ultraviolet absorption spectrum of the eluate determined on a recording spectrometer (Warren Spectracord as shown in Fig. 1, curve A).

*Results.* The eluted material was rechromatographed in the same system. No change in $R_f$ value was observed, but the eluate from the second chromatogram gave the absorption spectrum shown in Fig. 1, curve B.

Fig. 2 shows absorption spectra obtained from a pure sample of progesterone (100 γ) after each of 3 successive chromatographic runs under conditions similar to those used to separate the ovarian extract. Curve A, Fig. 1 is similar to curve A, Fig. 2, while curve B, Fig. 1 and curve C, Fig. 2 show close correspondence in detail. Savard *et al.* (6) have presented evidence that progester-

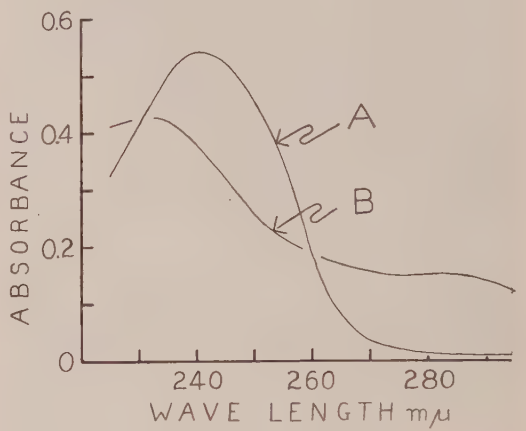


FIG. 1. (A) UV-absorption spectrum of eluates of spot on chromatogram of extract of ovaries of laying hens. (B) UV-absorption spectrum of same spot after previous spectrometry and re-chromatographing.

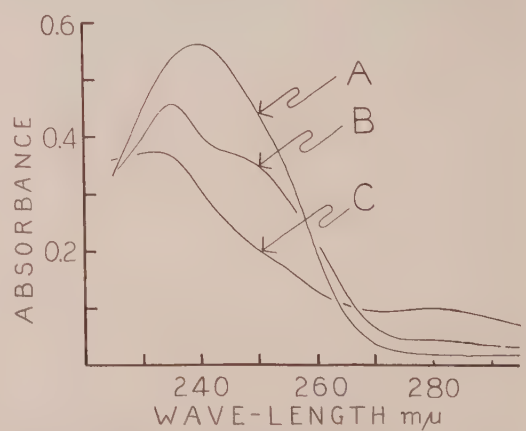


FIG. 2. (A) UV-absorption spectrum of reference progesterone after single chromatographic run. (B) Absorption spectrum after re-chromatography. (C) Absorption spectrum after further re-chromatography.

* Macdonald College Journal Series No. 401, supported by Research Grant from Nat. Research Council of Canada.


FIG. 3. (A) Spot on chromatogram carrying tracer amount of 21-$C^{14}$-labelled progesterone; stained with 2,4-dinitrophenylhydrazine and photographed using blue filter. (B) Radioautograph of same spot. (The photographs do not fully reproduce the detail of correspondence.)

one is broken down on filter paper by exposure to ultraviolet light. The absorption spectrum of the breakdown product is closely similar to that shown in curve B, Fig. 1, and curve C, Fig. 2. These results were interpreted as evidence that the compound isolated from avian ovaries was progesterone, and that changes in the absorption spectrum were due to chemical breakdown of progesterone under conditions of chromatography and ultraviolet spectrophotometry.

Another sample of the ovarian compound was mixed with 5 $\gamma$ of 21 –$C^{14}$-labelled progesterone and carefully rechromatographed without having been exposed to UV spectrophotometry. The strip was dried and then left in contact with X-ray film for 4 days to secure a radioautograph. The spot was then stained with 2, 4-dinitrophenylhydrazine. Under our conditions the 5 $\gamma$ of tracer progesterone gave a barely perceptible stain, whereas the labelled spot stained strongly. It will be seen from Fig. 3 that the radioautograph and staining of the spot showed close correspondence. This observation provides strong additional evidence for identity of the ovarian compound with progesterone.

We have failed to detect progesterone in blood of laying hens. However, extracts prepared by the method of Edgar(4) from 2 litres of blood from laying hens contained material which has an absorption spectrum similar to those shown in Fig. 1, curve B, and Fig. 2, curve C.

In other experiments the progesterone spot has been found on chromatograms of extracts of maturing follicles as well as of ruptured follicles. Although our results are mainly of qualitative significance, the degree of staining has suggested that the quantities of progesterone in the ovaries or follicles were small.

*Summary.* Progesterone has been detected on chromatograms of extracts of ovaries of laying hens and identification has been confirmed by radioautographic fingerprinting.

We are indebted to the National Research Council of Canada for grant-in-aid which made this work possible. One of us (R.H.C.) wishes to thank the Royal Society for a Nuffield Foundation Commonwealth Bursary which enabled this project to be initiated during a visit to the Nat. Inst. for Research in Dairying, Reading, in 1954. We wish also to thank Professor W. F. Oliver for his help in radioautography and Professor B. E. Baker for help with photography.

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Received January 15, 1957. P.S.E.B.M., 1957, v94.

# Lethality of Cell-Free Extract of *Candida albicans* for Chlortetracycline-Treated Mice.* (23002)

FRANK J. ROTH, JR.†, AND WILLIAM H. MURPHY, JR. (Introduced by J. T. Syverton)
*Department of Bacteriology and Immunology, University of Minnesota, Minneapolis.*

Action of an endotoxin in pathogenesis of *Candida albicans* infection has been suggested (1,2). This communication reports the lethality for mice of a cell-free supersonic extract of *C. albicans* in combination with chlortetracycline (Aureomycin, Lederle) and the comparative toxicity of viable *C. albicans* singly and in combination with chlortetracycline for guinea pigs, rabbits and monkeys.

*Materials and methods. Candida albicans,* strain 780, was grown on glucose-peptone-yeast-extract agar at 27°C for 72 hours, harvested, washed 3 times in cold (4°C) 0.85% NaCl solution (saline), and resuspended in saline to a 1% concentration by volume (15 x $10^6$ cells/ml). *Heat-killed cells* were prepared by immersion of the 1% viable cell suspension in a 58°C water bath for 90 minutes. *Mechanical lysate* was obtained by shaking a 2% saline suspension of cells with glass beads at 4°C for 2 hours in a paint-shaking machine; cellular debris was separated from lysate by centrifugation. Microscopic observation showed that 60-70% of the cells had been ruptured. *Supersonic extract* was made by exposing a 10% saline suspension of viable cells to supersonic vibration at 4°C for 45 minutes in a Raytheon Sonic

TABLE I. Lethality for Antibiotic-Treated Mice of Preparations of *Candida albicans.*

| Antibiotic | *C. albicans* preparation | Deaths at 96 hr* | Hr to 50% death |
|---|---|---|---|
| Chlortetracycline† | Viable cells‡ | 50/50 | 31 |
| — | *Idem* | 4/25 | |
| Chlortetracycline | Heat-killed cells | 17/30 | 96 |
| — | *Idem* | 1/20 | |
| Chlortetracycline | Supersonic extract, 0.5 ml | 50/50 | 10 |
| — | *Idem* 1.0 | 0/10 | |
| — | " 2.0 | 0/10 | |
| Chlortetracycline | Mechanical lysate | 8/30 | |
| — | *Idem* | 1/20 | |
| Chlortetracycline/10 g body wt | | | |
| 1 mg | — | 0/20 | |
| 2 | — | 5/20 | |
| 3 | — | 18/20 | 48 |
| 4 | — | 20/20 | 24 |
| Oxytetracycline | Supersonic extract | 2/10 | |
| " | — | 0/10 | |
| Chloramphenicol | Supersonic extract | " | |
| " | — | " | |
| Streptomycin | Supersonic extract | " | |
| " | — | " | |

* Mice were observed for 96 hr after inj.; data are accumulative totals from replicate experiments.

† Mice were prepared for inoculation of viable cells or cell preparations by prior inj. of 1 mg of antibiotic/10 g body wt. Levels of chlortetracycline *alone* inoculated for control purposes are given in the table.

‡ Viable cells were inj. as 0.2 ml of 1% stock suspension; 0.5 ml of killed cells, extract or lysate were used except in high level controls of the most active preparation (supersonic extract).

* Aided by grant from Division of Research Grants and Fellowships, N.I.H., U.S.P.H.S.

† Present address: University of Miami Medical School, Coral Gables, Fla.

TABLE II. Toxicity of Viable *Candida albicans* Cells for Animals Pretreated with Chlortetracycline.

| Test animal | Cells/100 g body wt* | Chl./10 g† body wt, mg | Survival of animals, days 0 | 1 | 2 | 3 | 4 | 10 |
|---|---|---|---|---|---|---|---|---|
| Guinea pigs | $15 \times 10^6$ | 1 | 4 | 2 | 0 | 0 | 0 | 0 |
| | " | | 2 | 2 | 2 | 2 | 2 | 2 |
| | — | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| Rabbits | $15 \times 10^6$ | 1 | 4 | 0 | 0 | 0 | 0 | 0 |
| | " | | 6 | 6 | 5 | 4 | 3 | 3 |
| | — | 1 | 4 | 4 | 4 | 4 | 4 | 4 |
| | $12 \times 10^6$ | .75 | 4 | 3 | 3 | 3 | 3 | 3 |
| | " | | 4 | 4 | 4 | 4 | 4 | 3 |
| | — | .75 | 4 | 4 | 4 | 4 | 4 | 4 |
| | $10 \times 10^6$ | .67 | 2 | 2 | 2 | 2 | 2 | 2 |
| | " | | 2 | 2 | 2 | 2 | 2 | 2 |
| | — | .67 | 2 | 2 | 2 | 2 | 2 | 2 |
| | $5 \times 10^6$ | .34 | 2 | 2 | 2 | 2 | 2 | 2 |
| | " | | 2 | 2 | 2 | 2 | 2 | 2 |
| | — | .34 | 2 | 2 | 2 | 2 | 2 | 2 |
| Monkeys | $15 \times 10^6$ | 1 | 4 | 0 | 0 | 0 | 0 | 0 |
| | " | | 2 | 2 | 2 | 2 | 2 | 2 |
| | — | 1 | 2 | 2 | 2 | 2 | 2 | 2 |

* *C. albicans* was inoculated intraper. into test animals; each ml of inoculum contained from $15 \times 10^6$ to $5 \times 10^6$ cells.

† Chlortetracycline (chl.) was inoculated intraper. into test animals prior to inj. with *C. albicans*.

Oscillator, Model S-102A, 9 Kc/s. The supersonic extract was freed from intact cells by filtration through a 02 Selas filter to yield a water-clear sterile filtrate. Microscopic examination revealed that very few cells had been ruptured by supersonic vibration. *Supersonic lysate* was obtained from cells recovered from Selas filters: a 10% suspension of cells was autolyzed by storage at 4°C for 24 hours, and the opalescent lysate separated from cellular debris by passage through an 02 Selas filter. All cell-free preparations were stored at –70°C in sealed ampoules until used. *Crystalline antibiotics* reconstituted in saline were used within 2 hours after preparation. Antibiotics were injected intraperitoneally (IP) 30 minutes before IP inoculation of *C. albicans* cells or cell preparations. American Dutch Belt rabbits (1.5-2.0 kg), monkeys (*Macaca cynomolgus* 2.5-3.0 kg) and guinea pigs (0.5 kg) were used to test toxicity of viable *C. albicans*. Adult albino mice were employed to assay the toxicity of viable or heat-killed suspensions of *C. albicans* cells and cell-free preparations.

*Results.* It is evident from the findings summarized in Table I that: (a) lethality for mice of chlortetracycline and viable *Candida albicans* cells was equaled or exceeded by that of combinations of the antibiotic with supersonic extract or lysate, while combinations with heat-killed cells or mechanical lysate were less effective; (b) action of the antibiotic was associated with chlortetracycline; oxytetracycline was only slightly active and chloramphenicol and streptomycin were inactive; (c) antibiotic, extract or lysate singly was not lethal for mice. The supersonic extract was most potent of all *C. albicans* preparations tested. The data compiled in Tables I and II show that a) infectivity and toxicity of viable *C. albicans* for animals pretreated with chlortetracycline were separable properties, b) toxicity of viable *C. albicans* cells in combination with chlortetracycline was demonstrated in use of four diverse animal species; c) rabbits in response to graded doses of cells and antibiotic required for manifestations of toxicity a minimal threshold inoculum of yeast cells.

*Discussion.* Analysis of host-parasite relationships in experimental candidiasis is complicated by the potentiative effect of aureomycin(3,4,5). Possible stimulation by aureomycin of *C. albicans* growth has been reported (6,7,8) which ultimately may cause displacement of the normal flora of the gastrointestinal tract. Winter and Foley(5) showed that aureomycin could inhibit hematogenous spread of *C. albicans* in mice and alter the distribution of lesions. The findings reported here may aid clarification of this complex host-parasite relationship. The factor extractable from *C. albicans* cells may be regarded as an endotoxin(9) potentiated by chlortetracycline, or alternatively, as an agent capable of enhancing subliminal toxicity of the antibiotic. Either possibility is of clinical significance.

The origin of the extractable *C. albicans* factor is suggested by consideration of the nature and biological potency of the various cell-free preparations tested. Mechanical lysate (heavily opalescent), resulting from rupture of 60-70% of cells, was only slightly ac-

tive; supersonic lysate (slightly opalescent) was more active; and supersonic extract (water-clear), which resulted from lysis of very few cells, was most lethal to chlortetracycline-treated mice. This inverse relation between cell rupture and extract potency may indicate that the extractable factor is liberated from the surface of *C. albicans* cells. Surface origin is compatible also with the activity of heat-killed cells.

*Summary.* Supersonic vibration of viable *C. albicans* cells released a substance lethal to mice treated with chlortetracycline. In the absence of antibiotic the cell-free preparation was inactive. The combination of supersonic extract with chlortetracycline was highly lethal to mice, that with oxytetracycline was slightly active, while that with chloramphenicol or streptomycin was inactive. Evidence was presented for the combined toxicity of viable *C. albicans* cells and chlortetracycline to diverse animal species.

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Received September 4, 1956. P.S.E.B.M., 1957, v94.

## Continuous Cultivation of Epithelial Cell Strain (FL) from Human Amniotic Membrane.* (23003)

JØRGEN FOGH AND ROSEMARY O. LUND. (Introduced by W. M. Stanley)
*Virus Laboratory, University of California, Berkeley.*

Several investigators have succeeded in establishing epithelial-like cell lines from normal human tissues. Chang developed subcultures of cells from conjunctiva, liver, appendix and kidney(1), Perry *et al.* cultivated a cell line derived from normal human skin (2) and Jordan carried human nasal cells in continuous culture(3). Primary cultivation of cells from human amniotic membranes in tissue cultures has been reported(4). Further work in this and other laboratories has shown amnion cultures to be suitable for studies of several viruses(5-7) and as a source of normal human tissue for research on cancer problems. This stimulated attempts to develop amnion cells in subculture. We report here successful continuous cultivation of a strain (FL strain) of human epithelial-like cells derived from normal amniotic membrane. They grew as good confluent monolayer cultures of clear flat cells on the glass surface in medium without embryo extract. Their growth rate was high and they were cultivated in media containing human serum as well as several animal sera. For tissue culture work in which use of first generation material is not essential, the use of this strain of cells in serial cultivation offers several practical advantages, namely elimination of difficulties involved in collecting fresh material and a long trypsinization procedure. Susceptibility of this cell strain to polio and APC viruses will be reported later(8).

*Material and methods. Placental tissue*† was handled as aseptically as possible at the delivery room. Disinfectants of "surface active" type were completely avoided during de-

* Aided by Am. Cancer Soc. Institutional Grant and Natl. Foundation for Infantile Paralysis.

† We wish to express our appreciation to Alta Bates Hospital, Berkeley, California, for providing placenta material.

livery. The whole placenta was placed in large sterile beaker and sent to this laboratory within a few hours. *Media and sera.* The following substances were used: 1. Trypsin (Difco, 1:250) at concentration of 1:400 in Tyrode's solution. 2. Versene (Disodium versenate, Versenes, Inc.) at concentration of 1:5000 in calcium and magnesium free phosphate buffer solution. 3. Lactalbumin hydrolysate (Nutritional Biochemicals). 4. Yeast extract (Difco). 5. Bovine albumin powder V (Armour). LY-medium is our designation for a medium consisting of Earle's balanced salt solution with 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 0.4% dextrose. Pooled human serum consisting of 16 pools from 8 donors and 7 pools from 30 donors has been used. The serum was not heat inactivated but filtered once through Selas filter (0.03 porosity) after mixing with basic medium. *Animal sera.* Ox and lamb sera were collected from slaughter houses as pools from 12 and 6 animals, respectively. Swine and horse sera were obtained from individual animals. In our work 2 pools of ox serum, 1 pool of lamb serum and serum from 1 swine and 3 horses were used. All animal sera were filtered once through Selas filter, heated at 56°C for one hour, and refiltered in basic medium before use.

*Technic for studies of cell multiplication.* The cultures were propagated in Povitsky bottles in LY-medium with 20% human serum for various lengths of time. Medium was removed and versene added to the bottle. After incubation at 37°C for several minutes the cells were completely removed from glass surface. Cells were sedimented from suspension in clinical centrifuge and then resuspended in small known volume of same medium. Cell concentration was determined by hemocytometer. The suspension was diluted to approximately 50,000 cells/ml and dispensed in test tubes, 1 ml/tube. Tubes were incubated at 37°C without fluid change. At 24-hour intervals during the following 7 days, 5 tubes/day were treated with versene in the same way and the average number of cells/tube calculated from hemocytometer counts. *Cultivation of cell strain.* The cells from which this strain was developed originated from a placenta delivered from healthy woman, Mar. 13, 1956. The amniotic membrane was stripped, stored overnight in 0.25% trypsin in Tyrode's solution, and processed the following day. After treating the tissue with trypsin in Tyrode's solution with magnetic stirring, a cell pack of 2.7 ml was obtained by light centrifugation. The cells were dispensed in different kinds of containers and media for various purposes. Cultures from which the FL strain originated grew in 50 mm Petri dishes in medium consisting of 20% ox serum and Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate. Each dish had been seeded with amount equivalent to 1/30 ml of cell pack, and in the following days incubated at 37°C in 4% $CO_2$ atmosphere. The preparations did not grow out to confluent cultures. On the 12th day, cells were transferred to test tubes with rubber stoppers. They were first treated with 3 ml versene at 37°C for ½ hour. At this time 1.5 ml trypsin was added/plate. After 15 to 20 minutes the cells were released from glass surface as single cells. Cells were sedimented from suspension by centrifugation, resuspended in LY-medium with 20% human serum and finally dispensed in a few tubes at very high concentration. At the next 2 weekly transfers the yield of cells obtained from enzymatic digestion decreased, and the cells were seeded in fewer tubes each time, until at 3rd transfer only one tube was seeded. After 3rd transfer the majority of cells had become vacuolated and enlarged. The medium was changed to 20% human serum in Earle's balanced salt solution with 0.5% lactalbumin hydrolysate and the cultures kept on this medium in the following transfers. From the 3rd to 6th transfer covering a period of 2 months, growth in the tube was patchy. The culture area was not increased until cells in 6th transfer started to grow more rapidly. At this stage the medium was changed to 20% human serum in LY-medium. The cells were now transferred to 2 tubes in which they grew out to confluency, and were then transferred to 4 tubes 3 days later. Since then the cells have been dividing

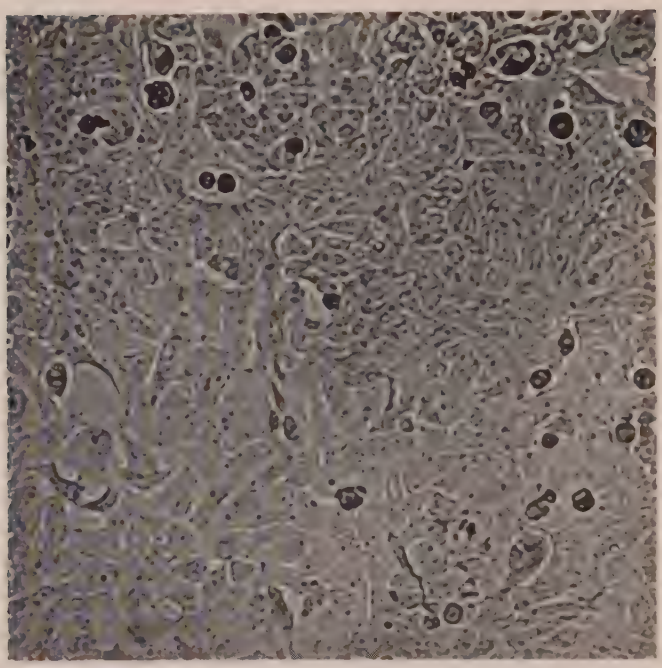

FIG. 1. Culture of FL cells in the 14. transfer. Grown on LY-medium with 20% human serum (100X).

rapidly. These FL cells have been cultivated continuously for 8 months, are now in their 30th passage and show an 8-10 fold cell increase within 7 days. Up to 10th transfer the procedure applied, necessary for complete removal of cells from glass surface, was the combined treatment of cultures with trypsin and versene. Since the 10th transfer all cells

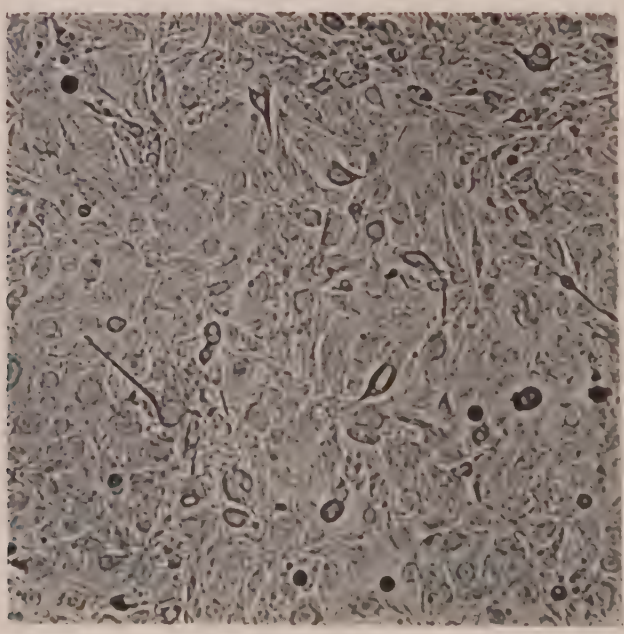

FIG. 2. Culture of primary amnion cells. Grown on LY-medium with 20% human serum (100X).

responded to treatment with versene alone and the trypsin was omitted subsequently. During this entire period of continuous cultivation, even at the time when growth was patchy, the cells kept their epithelial-like characteristics. During the stage when vacuolization and enlargement occurred, the cells became only slightly larger than originally. A fibroblastic-like stage was never observed.

*Results.* The serially propagated strain of amnion cells is shown in Fig. 1 as it appeared in the 14th transfer. It is being cultivated in tubes, plates and bottles of different sizes and forms uniform monolayer cultures of clear, flat, epithelial-like cells which in gross appearance are similar to cultures of cells directly isolated from an amniotic membrane (Fig. 2). Many mitotic cell forms were observed indicating high dividing activity of this cell strain.

*FL cells on different media. Media containing human serum.* Since June 14, 1956 FL cells have been grown in LY-medium plus 20% pooled human serum. At time of this writing (11/15/1956) they are in their 30th transfer. They were routinely subcultivated in this medium once a week with fluid change at 4-5 day. Test tubes, serum dilution bottles, 50 mm Petri dishes and Povitsky bottles were seeded respectively with 50,000, 1 million, 700,000 and 3 million cells and cultures were confluent within a week. Of 23 serum pools only one has shown a "toxic" effect on this cell strain and this appeared as a significant decrease in growth rate. This effect was not noticed on strains of HeLa cells cultured under identical conditions (Fig. 3). Attempts to reduce the amount of serum have so far resulted in slower growing cultures after a number of transfers. In LY-medium with 10% human serum cultures have been transferred 8 times within 2½ months with a dilution of 1:8 at the last few transfers, but the cells were somewhat granular and odd cell shapes were observed. Media containing 20% and 10% human serum in mixtures of bovine amniotic fluid and LY-medium have been tried and good cell growth has been observed for several passages.

*Media containing animal serum.* Cells

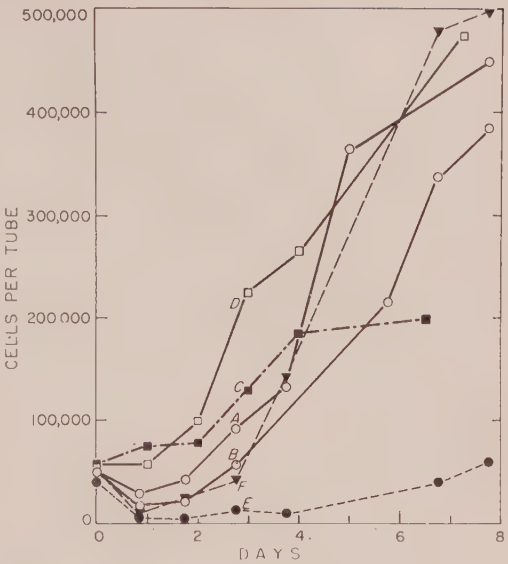


FIG. 3. Multiplication of FL and HeLa cells in LY-medium with 20% human serum. Curves A and B represent 2 different experiments in which FL cells from 7-day-old cultures were propagated in medium with serum from 2 different pools. C and D represent cells transferred from 3-day-old cultures and seeded in 1 and 2 ml respectively of conditioned medium. Curve E represents the effect of a "toxic" batch of human serum on multiplication of FL cells, while F represents growth of HeLa cells in the identical medium.

from 15th transfer in medium containing human serum were subcultured in media with animal sera. Sera from swine, lamb, horse and ox were tried in concentration of 20% in combination with different media. In the LY-medium all cells were kept alive and dividing for 6 transfers over 2½ months. At this stage however cultures grown on swine and lamb serum were eventually discarded, since they had become very granular and stopped dividing. Cells grown on ox and horse serum appeared to be healthy and they are at the present stage in their 11th transfer over a total period of 4½ months since cultivation in animal serum was started. In the last 3 passages the LY-medium has been modified by increasing dextrose concentration to 0.6% and sodium bicarbonate concentrations to 0.22%. Under these conditions the increase/week was 6-8 fold and cultures consisted of clear, normal looking cells. Cells grown in 10% ox serum in LY-medium gave the same appearance as those in 20% ox

serum in LY-medium but growth was slower (dilution 1:5 after 1 month). In LY-medium containing 0.5% bovine albumin powder and 5% ox serum, the cells were slow growing, but healthy. In media without serum, cells did not appear to divide. Cultures grown in test tubes in medium containing serum were washed several times to remove the serum. Several media without serum were tested for their ability to keep the cell sheet intact. In medium 199 and the LY-medium the sheet began to disintegrate within 5-7 days. Addition of a small amount of rabbit or chicken serum (2-4%) extended this period approximately 1-2 days.

*Multiplication studies.* The increase in cell concentration with time for FL cells under different conditions is shown in Fig. 3. The points represent number of cells seeded/tube at time 0 and average number of cells/tube counted in 5 tubes after various times of incubation.

When cultures were transferred at 7 day intervals the number decreased from 50,000 to 20,000 after the first day. Soon after the cells began to multiply and exceeded the initial count within 2 to 2½ days. From there on the cells multiplied rapidly with a generation time of 30 to 36 hours. At the 8th day the number reached was 400,000 to 450,000 cells/tube. Expressed as proliferation index (10), *i.e.* number of cells at end of 7 days divided by number of cells inoculated, a value of approximately 8 was obtained under these conditions. To avoid the initial decrease in number of cells, experiments were performed with cells transferred from young cultures and seeded in conditioned medium, *i.e.* medium in which they had already been growing for 3 days. By use of this technic there was no initial loss and cells multiplied rapidly, reaching a concentration of 200,000 and 475,000/tube at the 7th day. Apparently 1 ml of medium does not contain sufficient nutrient to support a high level of cell multiplication.

One pool of serum out of 23 showed toxic effect on FL cells although this serum did not affect growth of HeLa cells. During multiplication studies the pH of medium in which cells grew was measured simultaneously with each cell counting. The cells were normally seeded in medium of approximately pH 7.5; one day later the pH increased and rapid division of cells did not occur until the pH of medium by cell metabolism had been readjusted to 7.5°. It appears from a few experiments that it is possible to shorten this lag period by seeding the cells in a medium at lower pH. In this way an 18 fold increase in cell concentration has been obtained within a week using 3 day old cells seeded in 2 ml conditioned medium with pH of 7.0 and a fluid change after 3 days.

*Discussion.* The method of cultivation described differs from previous published methods in that this cell line was developed by trypsinization of the primary source and cultivated in a medium without embryo extract. Starting with a high number of cells the population diminished considerably during the first stages. Accordingly the area of growth on the glass surface was deliberately diminished so that the number of cells/area and / volume of culture fluid was still high. It seems likely that this method, in accordance with the hypothesis of Earle(9) helped the cells to adjust to continuous growth on a glass surface.

The appearance of cells has at all stages been epithelial-like. They did not grow out of predominantly fibroblastic cultures as was the case with the line of nasal cells described by Jordan(3).

The FL cell strain which has now been in large scale production for 5 months appears to be easily maintained in stock and divides rapidly enough to provide adequate amounts of cultures for production, diagnostic, or research purposes. Our main experience so far concerns cultures grown in medium containing human serum but good evidence has been obtained that this cell line can be carried on animal serum.

Multiplication studies of FL cells have revealed growth rates of the same order of magnitude as recently found for Chang's cells (10) although the conditions of cultivation are different. In our experience, the difficulties in obtaining growth promoting human

sera as described by Chang(1,11) has not been a problem. We have found that only one of 23 human serum pools was toxic to FL cells.

In preliminary experiments with FL and HeLa cells the FL cells have not produced tumors in treated rats using a technic‡ in which HeLa cells produced significant tumors. More work is needed however before a conclusion can be established.

*Summary.* A strain of human cells derived from a normal amniotic membrane has been cultivated in serial passage for 8 months in 30 transfers. This cell line was developed by trypsinization of the primary source and cultivated in a medium without embryo extract. The appearance of the cell has been epithelial-like at all stages of cultivation. Growth characteristics in media containing human serum or animal sera are reported. The cell strain has been grown in large scale.

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Received December 19, 1956. P.S.E.B.M., 1957, v94.

## Estrogen Antagonisms: Inhibition of Estrone-Induced Uterine Growth by Testosterone Propionate, Progesterone and 17-ethyl-19-nortestosterone. (23004)

RICHARD A. EDGREN AND DAVID W. CALHOUN
*Division of Biological Research, G. D. Searle and Co., Chicago, Ill.*

Recent literature has demonstrated a considerable interest in substances which antagonize the action of estrogenic compounds. Androgens have long been known to inhibit, at least in part, the effects of estrogens on various target organs(1). Progesterone, corticoids and various estrogenic substances also have been implicated (see 2 for review of literature). The reports generally leave much to be desired from a comparative standpoint as end points used vary from laboratory to laboratory and few workers have attempted more than qualitative analyses; thus adequate potency evaluations are difficult. The present communication discusses an estrogen-antagonistic action of 17-ethyl-19-nortestosterone (Nilevar) and compares activity of this substance to activities of testosterone propionate and progesterone, using estrogen-induced uterine growth as an index of activity.

*Materials and methods.* The estrone-stimulated uterine growth of intact, immature mice was employed for purposes of assay following essentially the procedure of Rubin, *et al.*(3). Our modification of this test has already been described(4). The response index was weight of uterus in mg. Five experiments were carried out using each of the 3 substances: progesterone, testosterone propionate and 17-ethyl-19-nortestosterone. A dose of 0.3 $\mu$g of estrone was employed as standard uterine growth-stimulator, against which the test compounds were assayed. In each experiment groups of 8-10 mice were treated with the estrone standard alone and estrone in combination with a series of doses

of test substance; an additional group of mice served as control receiving only corn oil injections. In analyses of data, it was assumed that a 100% inhibitor, when administered simultaneously with 0.3 μg of estrone, would produce a uterine weight equivalent to that of simultaneously run oil-treated controls. Thus for each experiment the difference between response obtained with 0.3 μg of estrone and that obtained with oil treatment was considered a 100% depression. Uterine weights of groups receiving inhibitor were compared to this total possible depression and percentage value obtained. In a typical test uteri of estrone-treated mice averaged 38.5 mg while oil control uteri averaged 9.8 mg; the difference, 28.7 mg, was considered 100%. A combined dose of 0.3 μg estrone plus 2 μg of 17-ethyl-19-nortestosterone, which gave an average response of 24.2 mg, was considered a 49.8% depression. Each experiment was calculated separately. These percentage depressions and logs of doses of antagonist were fitted with dose response lines using the method of least squares.

*Results.* All 3 substances produced marked depression of the estrone-induced uterine growth response (Fig. 1). Although the responses, as may be expected, were highly variable, the data supported the contention that 17-ethyl-19-nortestosterone was a potent inhibitor of estrone activity, being roughly 70 times as potent as testosterone propionate and about 50 times as potent as progesterone (Table I). Progesterone appeared to be slightly more active than testosterone propionate (about 1.4 times). Statistical analysis indicated adequate parallelness among the 3 slopes, so a single pooled value was used.

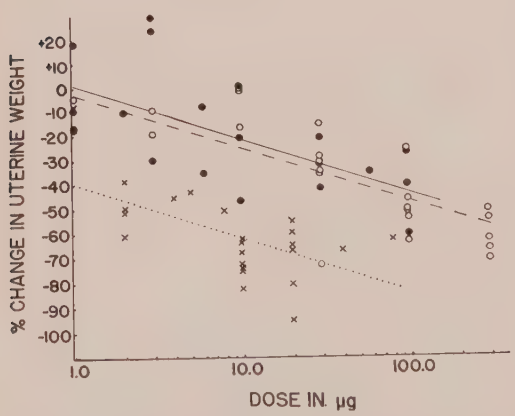


FIG. 1. Effects of testosterone propionate (dots and solid line), progesterone (circles and dashed line) and 17-ethyl-19-nortestosterone (crosses and dotted line) on estrone-induced uterine growth of intact, immature mice. 0% change = response of uterus to 0.3 μg of estrone; −100% change = control uterine level.

TABLE I. Regression Formulae of Uterine Growth Depression on Dose of Estrogen-Antagonist, and Potencies of Compounds Relative to Testosterone Propionate. y = % depression of uterine wt; x = log dose of antagonist. For definition of depression see text.

| Antagonist | Regression formula | Relative potency |
|---|---|---|
| Testosterone propionate | y = 1.04 − 22.20x* | 1.00 |
| Progesterone | y = − 2.28 − " | 1.41 |
| 17-ethyl-19-nortestosterone | y = −39.98 − " | 70.42 |

* b = −22.20 ± 2.93.

*Discussion.* 17-ethyl-19-nortestosterone is a synthetic compound with many properties similar to natural steroids: it has androgenic and anabolic properties, progestational activity, metrotropic activity, and estrogen antagonistic action. Comparisons among these are of interest. Saunders and Drill(5) showed it to be as active as testosterone propionate as anabolic agent when measured by levator ani response whereas it had only about 6% androgenic activity. 17-ethyl-19-nortestosterone is a progestational agent; it was more active than progesterone in the Clauberg assay (5-10 times), but a somewhat higher dose was required to maintain pregnancy in spayed rabbits(6).

Data on androgenic, anabolic and progestational activities show marked lack of correlation with estrone-inhibitory action of the compounds; in most studies 17-ethyl-19-nortestosterone was less active than testosterone propionate or progesterone, the Clauberg assay being an exception. Even in the latter test, however, the potency was considerably less than the estrogen-antagonistic action reported here. Therefore, estrogen antagonism appears to be a unique pharmacological property, distinct from androgenic, anabolic or progestational actions. All 3 substances have

metrotropic activity as measured by uterine growth in immature mice*, however, these uterine-growth stimulating effects were not marked at doses below 100 μg where estrogen-inhibitory activities were quite apparent. Progesterone and 17-ethyl-19-nortestosterone appeared to be less potent than testosterone propionate in stimulating uterine growth, and all 3 have dose-response curves with shallow slopes similar to those of so-called impeded estrogens(4,7). It is of particular interest that a number of compounds which have dose-response curves with extremely shallow slopes will antagonize the action of estrone on the uterus. Preliminary studies on the effects of 17-ethyl-19-nortestosterone as an antagonist of estrone-induced vaginal cornification suggested that keratinization of epithelial elements proceeds in a relatively normal manner, but that leucocytes are never absent from the smears(8).

*Summary.* Testosterone propionate, progesterone and 17-ethyl-19-nortestosterone are all antagonists of estrone-induced uterine growth in intact immature mice. Progesterone has about 1.4 times and 17-ethyl-19-nortestosterone has about 70 times the potency of testosterone propionate. This high activity of 17-ethyl-19-nortestosterone did not appear to correlate with the androgenic, anabolic, progestational or metrotropic properties.

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Received December 26, 1956. P.S.E.B.M., 1957, v94.

## Strains of Small Race *Entamoeba histolytica* Maintained in Outdated Blood Bank Blood Medium.* (23005)

RUSSELL MICHAEL MCQUAY, JR. (Introduced by Israel Davidsohn)
*Department of Pathology, Mt. Sinai Hospital and Medical Research Fn., Chicago, Ill.*

Interest has been growing in culturing strains of small race *Entamoeba histolytica,* especially since new strains can be isolated and maintained easily(1). Although strains of large race have been cultured in media containing whole or defibrinated blood or red blood cells from various sources(2-10), strains of small race have not been grown in whole human blood. Recently, Tarshis(11) prepared a blood medium for growing *Mycobacterium tuberculosis.* It is the purpose of the present paper to describe 2 modifications of this medium which have been adapted for the maintenance of strains of *E. histolytica,* particularly those of the small race.

*Materials and methods.* 1. *Diphasic medium.* Outdated blood bank blood approximately 4 weeks old was used and contained ACD solution as follows: citric acid U.S.P., 0.50 g, sodium citrate U.S.P., 1.37 g, and dextrose U.S.P., 2.45 g/100 ml of transfusion solution.† The medium was made as follows: For basal medium, heat to dissolve 6 g of agar-agar in 276 ml of distilled water containing 4 ml of glycerol. Autoclave at 15 lb pressure for 15 minutes. Store in icebox or use immediately after by cooling to 45°C in

* This work was supported in part by grant from N. I. of Allergy and Infectious Diseases, U.S.P.H.S.

† 480 ml of blood was collected in Baxter Transfuso-Vac bottles containing 120 ml of ACD solution.

water bath. Add immediately 4 ml of crystalline sodium penicillin G (160,000 units). Mix thoroughly; then add to mixture quickly 120 ml of 4-week-old blood which has been removed aseptically from the bottle by entering at "X" mark with appropriate syringes. Before the first entry into the bottle, briefly allow air to pass through sterile cotton-plugged needle plunged into "airway." Mix media thoroughly and dispense rapidly from sterile Kelly infusion bottle with bell-type filling attachment in 3.5 ml amounts into sterile screw-capped or cotton-plugged culture tubes. Make slants without butt and place them in incubator at 37°C for 24 hours to check for sterility. Overlay the slants with approximately 5 ml of buffered saline (pH 7.4) as previously described(1), by dispensing from a sterile Kelly infusion bottle supplied with bell-type filling attachment. Store media in icebox, and just prior to use bring it to room temperature and add a small amount of sterile Bacto-rice powder. 2. *Liquid medium.* The base for liquid medium was made as for the diphasic with omission of agar-agar. Blood, penicillin and 400 ml of sterile buffered saline were added and the medium dispensed in 6 ml amounts in sterile tubes. Rice powder was added, also. *Strains of E. histolytica tested.* The following strains of small race were carried on 48 hour schedule with 0.5 ml of sediment transferred: Griffith; Brown, a new strain isolated in July 1956 from stool specimen in charcoal slants; Conrad; and 110. K9, 200 and Komin large race strains were likewise maintained. The Komin strain was isolated from stool on charcoal slants in Sept. 1956. All strains were carried on egg slants(12) and in liquid charcoal cultures with 0.5 ml of sediment transferred every 48 hours. The egg slants served as controls. Isolation of new strains of large and small race from stools was not attempted on blood mediums.

*Results.* The strains of small race grown on egg slants and in liquid charcoal cultures multiplied well, but the majority of organisms were heavily vacuolated and rounded or crescent-shaped. Neither form showed much motility. The strains of large race, however, showed considerable motility in the same media.

In the diphasic blood medium all strains of small race showed exceptionally good growth with the greatest number of organisms in clumps of rice starch, and in starch which floated to top of the overlay. All strains of small race showed a decided increase in motility over the controls. The following are the subculture numbers for strains maintained in the diphasic blood medium: small races: Griffith-35; Brown-15; 110-17; Conrad-17; large races: K9-17; 200-17; Komin-24.

In the liquid blood medium, Griffith, Brown, and Komin, the only strains tested, showed sporadically good growth but these cultures were not maintained past the eighth subculture.

Generally, growth appeared greater in diphasic blood medium as compared to egg slant controls and liquid charcoal medium, but no comparative counts were made.

*Discussion.* The liquid medium was unsatisfactory for maintenance of strains of small race and large race as the red and white cells masked the trophozoites, especially small race trophozoites, many of which are not much larger than the red cells. In the diphasic medium, on the other hand, all strains tested showed excellent growth. Whereas in liquid charcoal medium and egg slants the small race trophozoite strains were non-motile or sluggishly motile, these same strains in diphasic blood medium showed increased degree of motility with many very actively motile forms. These actively motile forms often were difficult to observe unless they were in large clusters. It is interesting to note that amebae will grow so well in a medium to which old blood has been added.

*Summary.* 1. Two modified blood mediums, one diphasic, the other liquid, are described for growth and maintenance of *Entamoeba histolytica* of large and small races. The mediums are modifications of the one described and used by Tarshis(11) to grow *Mycobacterium tuberculosis.* 2. Strains of small race—Griffith; Brown, a new strain; Conrad; and 110—maintained in the diphasic medium exhibited increased motility as com-

pared to egg slant controls and increased growth over liquid charcoal cultures. The large race strains—200; K9; and Komin, a new strain—grew equally well in the diphasic blood medium. 3. The liquid blood medium was particularly unsatisfactory for maintenance of strains of small race, as the organisms were not easily discerned in the highly cellular sediment. 4. No attempts were made to culture stools or to isolate new strains of small or large race in either blood medium. 5. A 48-hour subculturing scheme was satisfactory for diphasic blood medium, liquid charcoal medium and egg slants; 0.5 ml of sediment was transferred.

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Received January 4, 1957. P.S.E.B.M., 1957, v94.

## Some Alterations in Serum Enzymes in Progressive Muscular Dystrophy.* (23006)

ARNOLD A. WHITE AND WALTER C. HESS

*Departments of Biological Chemistry and Neurology, Georgetown University Schools of Medicine and Dentistry, Washington, D.C.*

Considerable attention has been given recently to the increase in activity of serum glutamic oxalacetic transaminase associated with tissue destruction. Examination of rat tissues by Cohen and Hekhuis(1) and Awapara and Sealer(2) showed this enzyme to occur in highest concentration in heart muscle. Skeletal muscle has about 3/4, and liver about 3/5 of heart muscle activity. Thus it is not unexpected that in diseases involving these tissues, such as myocardial infarction (3), acute liver injury(4) and in several muscle disorders(5) there is an elevation of serum transaminase activity. Schapira *et al.* (6) found that in progressive muscular dystrophy there is an increase in serum aldolase. There are several possible explanations for the increase in serum enzyme concentration; muscle destruction, the enzyme may have its origin in tissue other than muscle, and acceleration of protein turnover may result in an increase of enzyme release, producing a new balance between serum enzyme and the mechanism for enzyme disposal. By concurrent determination of serum aldolase and transaminase we have attempted to examine the first two of these possibilities. In addition alkaline and acid phosphatases were determined as representatives of a third group of enzymes.

*Methods.* Serum glutamic oxalacetic transaminase was assayed to modification of the method of Karmen(7) developed by Steinberg *et al.*(8). Enzyme activity was expressed as micromoles of oxalacetic acid formed/hour/ml of serum at 37°. Serum aldolase was determined by the Dounce *et al.* (9) modification of the method of Sibley and Lehninger(10). Following the suggestion of Beck(11) we extended chromogen reaction time from 30 to 60 minutes. The substrate employed was the magnesium salt of fructose

* This study was aided by grant from Muscular Dystrophy Assn.

TABLE I. Comparison of Mean Values of Enzyme Activity in Normal Serum and Serum of Muscular Dystrophy Patients.

| | Children | | | Adults | | |
|---|---|---|---|---|---|---|
| Enzyme | Dystrophy 8, age 7-13 | Control 10, age 2-14 | p | Dystrophy 9, age 15-54 | Control 6, age 22-40 | p |
| Alkaline phos., mM/l/hr | 3.5 ± .80 | 3.3 ± 1.62 | * | 2.2 ± 1.46 | 2.1 ± .59 | |
| Acid phos., mM/l/hr | .79 ± .18 | .64 ± .30 | | .64 ± .20 | .44 ± .06 | .05 |
| Aldolase, μg p/ml/min. | 1.82 ± .43 | .28 ± .10 | .001 | .55 ± .50 | .19 ± .05 | .001 |
| Transaminase, μM/ml/hr | 2.59 ± 1.80 | .85 ± .27 | .01 | .86 ± .42 | .65 ± .12 | .05 |
| $\frac{\text{Aldolase} \times 10}{\text{Transaminase}}$ | 5.6 ± 2.36 | 3.4 ± .79 | .01 | 6.3 ± 3.49 | 2.8 ± .30 | .05 |

* Where no value for p for difference is given, there was no significant difference.

diphosphate (Mann). It was used without further purification. At normal serum aldolase levels 1 ml of serum need be used, however with higher activity 0.5 ml is sufficient. Activity was expressed as μg of triose phosphate phosphorus formed/minute/ml of serum at 37°. Alkaline phosphatase was determined by the method of Bessey *et al.*(12), using 0.1 ml of serum. The acid phosphatase method used was that of Andersch and Szcypinski(13). Phosphatase units are expressed as millimoles of p-nitrophenol/liter of serum/hour.

*Results.* The cases were divided into 2 groups, adults and children. Average values, standard deviations and significance of differences between results in the 2 groups and the controls are given in Table I. Considering first the serum alkaline and acid phosphatase determinations, (Table I), there appears to be little or no alteration produced by muscular dystrophy. Our control sera were from adult laboratory personnel, and from hospitalized children with no neuromuscular disease and with aldolase and alkaline phosphatase activities within normal range(6,12). No information was available on normal range of acid phosphatase in children by the method we employed.

Our results for serum aldolase activity agree with the much larger series of Schapira *et al.*(6). In 61 cases of childhood muscular dystrophy they found a serum aldolase range of 0.3 to 13.0 with a mean of 3.3. We found a range of 0.37 to 4.08 with a mean of 1.82. Their values on normal children, mean 0.4 and range 0.2 to 0.6, are also in agreement with ours. On adult cases of muscular dystrophy we also found elevation in serum aldolase as reported by Schapira *et al.* Childhood muscular dystrophy is accompanied by higher serum aldolase activity than in the adult. This is in accord with previous observations(6,14). Siekert and Fleisher(5) determined serum transaminase on 3 cases of muscular dystrophy, presumably adults. They found an elevation of activity in 2 of them. Their controls consisted of 25 men and 25 women. The mean for females was 0.85 ± 0.177 and for males 1.16 ± 0.320, for the combined males and females 1.01 ± 0.298. They considered abnormal those activities that exceeded the mean plus 3 standard deviations. No adults with muscular dystrophy in our series can be considered abnormal by their criteria. Our own smaller control series had a lower transaminase activity range than that of Siekert and Fleisher. However there was no significant difference, $p = 0.05$, between means of control series and adult dystrophy cases. The mean activity for dystrophic children was 2.59 which is well above the normal range of either our own controls or the adult series of Siekert and Fleisher. There is a significant difference, $p = 0.001$, between the means for dystrophic and normal children.

The level of serum transaminase appears to be proportional to amount of heart muscle damage in myocardial infarction(15) and liver injury(16). It appears probable that elevated serum transaminase in muscular dystrophy similarly comes from skeletal muscle damage. Examination of data in Table I indicates there is a correlation between aldolase and transaminase activity, as aldolase

TABLE II. Aldolase and Transaminase Activities of Rat Tissues.

| | Aldolase | Ref. | Transaminase | Ref. | Aldolase × 10 / Transaminase |
|---|---|---|---|---|---|
| Skeletal muscle | 3440 mg/kg/min. | 10 | 289 μM/g/hr | 2 | 119.2 |
| Heart muscle | 620 | 10 | 334 | 2 | 18.5 |
| Liver | 560 | 10 | 250 | 2 | 22.4 |
| Serum | 2.8 mg/l/min. | 18 | 11.1 μM/ml/hr | 16 | 2.5 |

activity increases so does transaminase. This suggests that increased aldolase in muscular dystrophy is also derived from muscle.

It appeared of interest to calculate a factor that expressed the relative activities of serum aldolase and transaminase. This was done by multiplying the units of aldolase activity by 10 and dividing the product by transaminase activity. Our normal adult series had an A/T ratio ranging from 2.5 to 3.3, mean 2.8, Table I. The control children had an A/T ratio of 2.3, range 1.9 to 4.7. Relative to these controls, 13 of 17 muscular dystrophy cases showed elevations of the A/T ratio. The elevation was significant, $p = 0.001$, in the case of children but not in adults. In contrast to this are the results from the study of myocardial infarction by Siegel and Bing (17). They determined aldolase and transaminase in 5 cases of myocardial infarction and in 9 normals. We have recalculated their results to units we employed and the mean A/T ratio of their normals was 3.6, which is comparable to our A/T ratio of 2.8. From their data one finds that in myocardial infarction the A/T ratio falls below normal, in one case reaching 0.7. This suggests that in myocardial infarction more transaminase than aldolase is being released into the blood.

Concentrations of aldolase and transaminase in human tissues are not known. From several sources was assembled a Table of the aldolase and transaminase activities of rat tissues, recalculated to the same units used here (Table II). From these data it would appear that simply rupturing skeletal muscle cells would increase the A/T ratio of serum because of higher A/T ration of muscle, if it is assumed that both are cleared at comparable rates. Another source appears unlikely, especially since phosphatases, that do not occur in high concentrations in muscle, are not elevated, as might happen, for example, if liver, in which there is a high concentration of phosphatases, was the source for the extra aldolase.

An alternative explanation would be that aldolase and transaminase are removed from plasma at different rates. Sibley and Lehninger(18) confirming the observations of Warburg and Christian(19) found aldolase to be removed rapidly from plasma of rats. They injected crystalline aldolase and found serum aldolase elevated about 7 fold within 15 minutes, while 12 hours later the activity had fallen to only 1½ times normal value. Comparable experiments with transaminase have not been done. It appears, however, that transaminase is disposed of readily, since unless blood is sampled within 3 days after myocardial infarction, elevation in transaminase activity may be missed(20). The low A/T ratio in myocardial infarction may reflect the fact that the A/T ratio in heart muscle is only 1/6 of that found in skeletal muscle. Further data are needed from experiments in which human tissues and serum are analyzed by the same methods.

*Summary.* Acid and alkaline phosphatases, transaminase, and aldolase were determined in sera of 8 children and 9 adults afflicted with progressive muscular dystrophy. There was no alteration in phosphatase activity. Aldolase was increased in both children and adults while transaminase was distinctly elevated only in children. The ratio of aldolase to transaminase activity in serum increased in muscular dystrophy. The results indicate that increased activities of both aldolase and transaminase in progressive muscular dystrophy are derived from diseased muscle.

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Received January 9, 1957. P.S.E.B.M., 1957, v94.

## Pressor and Myotrophic Activities of Incubated Plasma.*† (23007)

G. M. C. MASSON, G. HELMCKE,‡ A. C. CORCORAN AND IRVINE H. PAGE
*Research Division, Cleveland Clinic Foundation and Frank E. Bunts Educational Institute, Cleveland, O.*

Plasma from children in the hypertensive phase of acute glomerulonephritis was reported to have pressor activity which diminished or disappeared during recovery(1). These samples had been at room temperature for sometime; activity was increased by incubation at 37°C for an additional 12 hours, a procedure which elicited similar, but less pressor activity in samples from normal children. The possibility was raised that the pressor agent might be angiotonin, liberated because the plasma of hypertensive subjects contained increased amounts of renin.

Accordingly, a survey was done in which we tested in Dibenamine-treated, anesthetized rats, or pithed rats, the pressor activity of plasma from 28 patients with established hypertension, 14 patients in malignant phase of essential hypertension, 3 with hypertension proven to be of renal origin, 7 with hypertension and chronic glomerulonephritis and 6 normotensive patients with renal disease of systemic lupus erythematosus as well as 11 normal subjects. For these tests, blood was taken by Dr. Carlos Nijensohn under sterile conditions into heparin-containing tube, immediately centrifuged, plasma separated and divided in 2 fractions. One fraction was incubated for 24 hours at 37°C and the other frozen. Both were assayed at the same time, using angiotonin as the reference standard for pressor activity. Freshly frozen plasma in doses to 0.2 ml gave irregular and small pressor responses similar to those elicited by the same volume of saline. The incubated samples were all pressor, approximately in the same degree and without reference to presence of hypertension. In brief, the survey indicated that pressor activity in question, while apparently not of immediate significance in problem of hypertension, represented at least an interesting property of shed, pre-

* This study was supported in part by grant from Cleveland Area Heart Soc. and was preented at the annual meeting of the Amer. Physiol. Soc., 1956.

† Dr. Merlin Bumpus graciously performed the physico-chemical studies.

‡ Now resident at Port au Prince, Haiti.

served human blood. Further studies were undertaken which are summarized in this report.

*Procedures.* Pressor activity was tested by intravenous injection into female rats, 150-200 g body weight. In early experiments these were pithed(2); it was subsequently more satisfactory to prepare them by Amytal anesthesia and subcutaneous injection of 3 mg of pentolinium in solution in 20% polyvinylpyrrolidone. The ganglion-blocker stabilizes blood pressure of the anesthetized rat and also enhances pressor responsiveness(3). Myotrophic properties of incubated plasma were tested in parallel with the frozen fractions, on guinea-pig ileum, rabbit duodenum, rabbit and rat uterus and rabbit aortic strips (4) in comparison with angiotonin and with epinephrine. Most of pressor and myotrophic assays were semi-quantitative, with an accuracy of about 50 to 150% of standard.

*Results.* (a) Incubation. Samples of both human and dog plasma were incubated for varying periods to 5 days, fractions being removed at intervals for freezing and subsequent assay. The maximum pressor activity was reached between 24 and 48 hours of incubation at 37°C and this remained constant thereafter in both incubated and frozen samples. This maximum was equivalent to 0.8 to 2.0 units(5) of angiotonin/ml; one unit is approximately equal to 1 Indianapolis unit or 1/5 Goldblatt unit. Incubated serum also showed pressor activity, although this demonstration was complicated by serotonin in the control sample.

(b) Site of Sampling. Simultaneous venous and arterial samples from dogs and rats were about equally active, with a suggestion of some greater activity in venous samples. Dr. F. Mason Sones kindly collected samples from children during diagnostic cardiac catheterization; these came from the supra- and infra-renal portions of the inferior vena cava. Dr. Eugene Poutasse graciously obtained samples during renal operations from renal and peripheral veins. These simultaneously collected pairs of samples were inactive before incubation but equally active after incubation, indicating that the ultimate origin of the pressor activity might not be renal.

(c) Myotrophic activity. Incubated samples of plasma caused contraction of rabbit aorta, guinea-pig ileum, rabbit and rat uterus. The direction, latency and duration of these responses more closely resembled those which occurred after application of angiotonin than those due to epinephrine; epinephrine produces relaxation in the rat uterus and contraction in rabbit uterus, while angiotonin contracts both preparations. In the case of rabbit duodenum, epinephrine decreased amplitude of contraction, angiotonin increased tone and incubated plasma provoked an initial decrease in amplitude followed by recovery of contraction and increase in tone. Dr. Leal Prado (then at Mount Sinai Hospital, Cleveland) kindly tested incubated plasma on the frog Laewen-Trendelenburg preparation and found it to be vasoconstrictor.

(d) Nature of agent. The pressor activity was not affected by treatment of rats with Dibenamine or Dibenzyline, by mixing the plasma with sodium thioglycollate or by giving rats bromolysergic diethylamide, confirming the conclusions of Dekanski(6) that it is not an adrenergic amine, not Pitressin, serotonin or, since it is not depressor, histamine.

The suggestion that it might be angiotonin was tested in several ways. First, angiotonin was added to plasma and the mixtures incubated; pressor activity of added angiotonin disappeared in 2-3 hours, at which time activity of the pressor substance formed on incubation was not yet detectable. Next, the pressor activity was not enhanced by adding renin-substrate to plasma prior to incubation. Samples of serum of monkeys immune to renin were obtained from Dr. George Wakerlin and incubated, as were also plasma of rats made resistant to renin by repeated daily injections of renin; these samples, presumably containing anti-renin, yielded full pressor activity on incubation. Renal origin of activity was also excluded by demonstrating pressor activity in samples of plasma from 24-hour nephrectomized rats. Lastly, incubated plasma was precipitated with 55% alcohol

at room temperature and the supernatant, after removal of alcohol, was incubated with pepsin, trypsin and chymotrypsin under conditions which rapidly destroyed the activity of control samples of angiotonin; in contrast samples of incubated plasma retained pressor activity.

Further preliminary experiments showed that the activity is not destroyed by standing at room temperature during 9 days or by boiling for 15 minutes, that it is not dialyzable, that it is soluble in 55% alcohol but that the supernatant from plasma rapidly mixed with 4 volumes of alcohol was inactive.

*Discussion.* The experiments show formation in incubated plasma and serum of an agent which is pressor in intact rats, is vasoconstrictor and directly myotrophic. While these activities correspond to those of angiotonin, this material is not ultimately of renal origin, does not have the properties of angiotonin(7) nor of other myotrophic polypeptides with regard to proteolytic enzymes(8). The experiments do not further elucidate its nature. They suggest that, as a widely distributed property of mammalian blood, it may have ultimate physiological significance and should be of concern in procedures which involve the storage of blood, plasma or serum.

Since our preliminary communication on this substance, 3 articles have appeared which may bear on its nature. Gabr(9) describes isolation from plasma of a long-chain, C18 unsaturated fatty acid, tentatively identified as 3-octodecenoic acid; this substance is possibly a product of phospholipid breakdown and, in low concentrations, causes a slow contraction of isolated jejunum of the guinea pig. More recently, Titus, Weiss and Hadju (10) isolated palmitoyl lysolecithin from plasma and tissues and have characterized its activity as digitalis-like, noting that a large part of the material is present in inactive form. Gayer and Sarre(11) have described a high-molecular weight, acetone- and alcohol-soluble acid which is vasoconstrictor and is present in normal human plasma and, in apparently greater amounts, in plasma of patients with essential hypertension. These observations indicate that there is a group of myotrophic lipid agents with which the above described pressor activity may eventually be identified. The fact that this activity develops during incubation *in vitro* suggests that like palmitoyl lysolecithin, it may exist in active and inactive forms, the latter subject to slow, presumably enzymatic degradation in plasma.

*Summary.* Incubation of normal human, dog or rat plasma at 37°C for 12 or 24 hours unmasks a pressor and myotrophic activity which is not due to known vasoactive substances.

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Received January 11, 1957. P.S.E.B.M., 1957, v94.

# Duodenal Fistula Dog as Test Animal for Acid-Stable or -Labile Compounds.* (23008)

CHENG-CHUN LEE. (Introduced by K. K. Chen)
*The Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.*

The pharmacologic assay of certain orally administered drugs is handicapped by the fact that the drugs are partially or completely destroyed by the gastric acids. In order to overcome this difficulty, duodenal fistula dogs have been prepared for the testing of such compounds. The procedure is described in this communication.

*Material and methods. Duodenal fistula dog.* Healthy female mongrel dogs were anesthetized with an intravenous dose of 30 mg of sodium pentobarbital per kg body weight. The inner part of a Lucite Thomas intestinal fistula(1) was placed into the duodenum about 2 inches from the pyloric opening; the outer part was inserted in a lateral abdominal incision about one inch to the right of the mid-line and one inch from the last rib. Five fistula dogs† weighing between 10.2 and 16.5 kg, were used in these studies. They were operated on 44, 40, 7, 4 and 2.5 months prior to these experiments. *Testing material.* The antibiotic erythromycin‡ was chosen because it possesses properties that make it especially suitable for the tests: First, erythromycin is absorbed through the gastrointestinal tract (2). Second, erythromycin B is more acid-resistant than erythromycin A(3); both were used in these studies for comparison. Third, the serum and urine concentration of erythromycin can be readily assayed by a cup plate method(4). The erythromycin A used in these studies assayed 956 μg/mg, and the erythromycin B assayed 637 μg/mg. They were given in capsules on weight basis. All dogs were starved overnight and single doses of erythromycin administered either by mouth or through the fistula. Each animal received water by stomach tube, 25 ml/kg at the beginning of the experiment and 10 ml/kg 2 hours later. This arbitrary procedure was to insure sufficient output of urine. At intervals, blood was drawn from the jugular vein and urine was collected in graduated cylinders at the end of the second and the sixth hour. Erythromycin was assayed on the serum and on the urine appropriately diluted with physiological saline, against standard curves based upon horse serum and physiological saline, respectively. The dogs were allowed to rest for one week before the experiments were repeated.

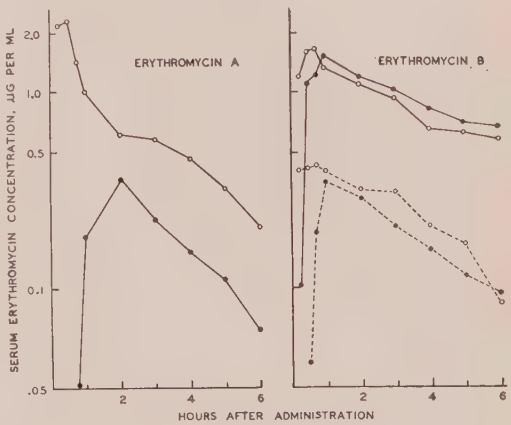


FIG. 1. Serum erythromycin levels following oral (dots) and intraduodenal (open circles) administration. Solid lines, 20 mg/kg; interrupted lines 5 mg/kg.

*Results.* The average erythromycin serum levels of 5 duodenal fistula dogs are shown in Fig. 1. After intraduodenal administration of 20 mg/kg of erythromycin A, the serum level reached a peak within 30 minutes. It dropped rapidly during the first hour and declined rather slowly thereafter. After the same oral dose, on the other hand, the serum level was much lower, indicating considerable destruction of erythromycin A in the stomach. In this case, the peak of the serum level was not reached until about 2 hours after admin-

* The author wants to thank R. O. Froman for his technical assistance; D. W. Ziegler and Stanis Stroy for penicillin assays.

† The cystic duct of these dogs was tied off with braided silk during operation for the purpose of hepatic bile collection in other studies.

‡ The trademark of Eli Lilly and Co. for the antibiotic erythromycin is 'Ilotycin'.

TABLE I. Urinary Recoveries of Erythromycin 6 Hours after Administration.

| Erythromycin | Dose, mg/kg | % of dose* Oral | Intraduodenal |
|---|---|---|---|
| A | 20 | 2.4 ± .9 | 7.6 ± 1.3 |
| B | 20 | 15.9 ± 2.0 | 9.8 ± 1.6 |
| B | 5 | 9.5 ± 1.0 | 12.6 ± 2.2 |

* Avg and stand. error.

istration and the serum concentration at the end of 6 hours was only one-fourth of that obtained after intraduodenal administration.

When erythromycin B was given to these 5 duodenal fistula dogs, the serum concentrations reached about the same peak after oral and after intraduodenal administration. After oral administration of 20 mg/kg, the maximal serum concentration was obtained at one hour, whereas the peak was reached about 15 minutes earlier when the drug was administered into the duodenum. In both cases, the serum erythromycin concentration declined slowly thereafter. Oral and intraduodenal administrations of 5 mg/kg of erythromycin B resulted in identical serum concentration curves. This clearly demonstrates that erythromycin B is acid-resistant.

The urinary recoveries of erythromycin A and B are summarized in Table I. An average of 2.4 ± 0.9% of erythromycin A was recovered 6 hours after oral administration and an average of 7.6 ± 1.3%, after intraduodenal administration. Much larger recoveries were obtained with erythromycin B, 9.5 to 15.9% of the dose when given orally and 9.8 to 12.6% when administered to the duodenum. This again shows that the gastric acids have no apparent effect on the absorption of erythromycin B given orally.

*Discussion.* The use of duodenal fistula dogs as test animals for acid-stable or -labile compounds has many advantages and is practical. First, an unanesthetized animal is used. Second, a compound can be given to the same dog orally or intraduodenally. Third, the fistula dogs can be used again and again. Fourth, by administration to the duodenum through the fistula, it can readily be determined whether a compound is absorbed through the intestinal tract at all. Fifth, the effectiveness of a special coating agent for acid-labile compounds can be checked. Sixth, the usefulness of various buffer agents added to acid-labile compounds can be tested.

*Summary.* The usefulness of duodenal fistula dogs as test animals for acid-stable or -labile compounds is demonstrated and the practical application is discussed.

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Received January 18, 1957. P.S.E.B.M., 1957, v94.

# Studies on Amino Acid Inadequacy of Blood.* (23009)

Henry S. Perdue, G. Frederick Lambert, and Douglas V. Frost
*Abbott Laboratories, North Chicago, Ill.*

Azotemia is a clinical sign of massive gastrointestinal bleeding(1-6). Several interesting studies relate digestion of large amounts of hemoglobin to increased blood NPN in the absence of shock, dehydration, starvation, or impaired renal function(1,5,7). Catabolism of large amounts of any protein may be followed by an increase in blood urea(8). Hemoglobin feeding, however, appeared to produce a proportionately greater increase in blood urea of dogs than casein and ground beef(9). Deficiencies of certain amino acids also increase blood NPN(10-12). This suggests that the known amino acid imbalance of blood(13) contributes to the azotemia. These studies further evaluate human blood and plasma and canine blood as sources of essential amino acids for protein depleted adult male rats. They also explore, in a preliminary way, the relationship of amino acid imbalance to azotemia following ingestion of large amounts of blood.

*Experimental.* The procedure used by Frost(13) was modified for protein repletion experiments. Young male rats of the Abbott stock weighing 150-200 g were prepared for assay by 12-day depletion on a low-protein diet (NP 14).† They were then oriented to drinking protein solution by a 3-day drink trial with a modified fibrin hydrolysate.‡ The rats were again depleted for 3 days and divided into groups. The citrated blood or plasma (Formula B, U.S.P. and N.I.H.) for each experiment was pooled. Where additions were made on a nitrogen basis, suitable aliquots were analyzed for Kjeldahl-nitrogen. Blood was then diluted with water and solutions of DL-methionine and L-isoleucine so 18 ml of final solution contained 120 mg of blood-N. When blood was not diluted, the additions were blended with the blood in a Waring blender. Enough solution was prepared to last the term of each experiment. All solutions were stored in the refrigerator. During preparatory and assay periods the rats had access to low-protein diet and deionized water. The rats were allotted 120 mg N/rat/day unless otherwise stated. The volume of solution taken daily by each rat was recorded and the rats were weighed at suitable intervals about 20 hours after the preceding feeding. Group variations were expressed as standard error (S. E. $= \sqrt{Sx^2/n(n-1)}$). In the rat experiments on blood urea-N 300 g males of Abbott stock were fasted 17 hours. Then citrated human blood or blood supplemented with 1.15 g of L-isoleucine and 0.31 g of DL-methionine/100 ml was administered by gastric intubation under light ether anesthesia. The rats which ingested about 24 ml of blood received 2 doses with one hour elapsing between initial 14 ml dose and final 10 ml dose. Time was counted after initial intubation. The rats receiving about 14 ml of blood were fed only once. Similar fasted and anesthetized rats were the controls. One ml oxalated blood samples were obtained by heart puncture for urea-N determinations(14) and normal feeding was not allowed until one sample was removed from each rat. A similar experiment employed groups of 2 dogs each. The dogs fasted for 2 days and then received lyophilized dog blood and citrated human blood at daily intakes of 300 and 375 mg N/kg body weight respectively for 11 days. A non-

* This work was reported to Am. Inst. of Nutrition, Atlantic City, Apr., 1956.

† NP 14: Sucrose, 85%; Jones and Foster Salt Mixture (28), 4; Agar, 1.4; hydrogenated vegetable oil, 4; corn oil, 4; Dry vit. A and vit. D mixture (8000 I. U. vit. A and 1500 I. U. vit. D/g) .1; choline chloride, .1. Vitamins, mg/100 g of diet: thiamine hydrochloride, .6; Ca DL-pantothenate, 5; inositol, 5; folic acid, .3; menadione sodium bisulfite, .1; vit. $B_{12}$ concentrate (3 mg vit. $B_{12}$/g) .1.

‡ Aminosol (trade mark), 5%, Abbott Labs., N. Chicago.

TABLE I. Protein Repletion by Human Blood and Plasma (120 mg N/Rat/Day; 6 Rats/Group).

| N source | Route | Supplement, % (W/V) Iso-leucine | Methio-nine | Avg 10-day gain, g ± S.E. | % allotment consumed |
|---|---|---|---|---|---|
| Blood | Tube | | | − 9 ± 2.02 | 100 |
| | " | .7 | .45 | 14 ± 2.6 | 100 |
| | Oral | | | −18 ± 2.7 | 18 |
| | " | .7 | .45 | 30 ± 1.4 | 100 |
| Plasma | Oral | | | 11 ± 2.5 | 77 |
| | " | .32 | | 31 ± 1.9 | 100 |
| | " | .32 | .19 | 41 ± 1.7 | 100 |
| Fibrin hydrolysate | Oral | | | 33 ± 2.0 | 100 |

TABLE II. Isoleucine and Methionine Deficit in Blood (Protein Repletion; 5-9 Rats/Group).

| Exp. | Source of blood | Supplement, mg/120 mg N Iso-leucine | Methio-nine | Avg 10-day gain, g ± S.E. | % allotment consumed |
|---|---|---|---|---|---|
| I | Canine | | | −11 ± 1.1 | 21 |
| | | | 30.4 | −14 ± 1.4 | 20 |
| | | 41.6 | | − 2.4 ± 1.4 | 57 |
| | | 41.6 | 30.4 | 33 ± 1.3 | 100 |
| I | Human | | | −16 ± 1.0 | 10 |
| | | | 20.3 | −14 ± 2.0 | 10 |
| | | 43.7 | | 11 ± .9 | 100 |
| | | 43.7 | 20.3 | 22 ± 1.7 | 100 |
| II | Human | 22 | 20.3 | − 3 ± 1.9 | 85 |
| | | 33 | " | 7.5 ± 3.3 | 89 |
| | | 44 | " | 16 ± 1.8 | 100 |
| | | 55 | " | 20 ± 1.4 | 97 |
| | | " | 10 | 13.3 ± 1.1 | 100 |
| | | " | 15 | 17.3 ± 1.4 | 100 |
| | | " | 25 | 17.7 ± 1.8 | 100 |
| | | " | 30 | 21.8 ± 1.4 | 100 |

protein diet mixture (NP 13X)§ supplied the other known nutrients and 70 calories/kg body weight/day. Blood and non-protein diet were fed at 9 a. m., 1 p. m., and 3 p. m. Blood samples were taken daily at 8:45 a. m. and on the eleventh day an additional sample was taken at 4 p. m. Blood samples were analyzed for urea(15) and NPN(16) and nitrogen balance of the dogs was followed. After 11 days the dogs were fed 150 mg N/kg/day as beef fibrin for 3 days and were then given 150 mg N/kg/day as human blood for 4 days. The blood and non-protein diet were fed at 9 a. m. and 3 p. m. and blood urea and NPN and nitrogen balance were followed as before. Amino acid analyses were performed on hydrolyzed human blood and plasma and dog blood. Microbiological assays(17,18,19) were performed after refluxing with excess 5 N hydrochloric acid for 5-6 hours. Samples for chemical tryptophan analysis(20) were refluxed with 5 N sodium hydroxide for 5 hours. Additional samples were analyzed for Kjeldahl-nitrogen and all analyses were calculated as grams of amino acid/16.0 g N of the original materials. Three samples of human blood, and 2 samples of human plasma and dog blood were analyzed and the results averaged.

§ NP 13X: Sucrose 69.4%; hydrogenated vegetable oil 18; corn oil 3; Oladol (U.S.P. units/g: 55,000 A and 5500 D); .5; salt mixture(28) 4; agar 2.50; choline chloride .30; wheatgerm oil .25; liver fraction "O" .2. Vitamins, mg/100 lb of diet: inositol 2270; p-aminobenzoic acid 45; thiamine hydrochloride 272; riboflavin 272; nicotinamide 5450; pyridoxine hydrocloride 182; Ca DL-pantothenate 917; folic acid 50.

TABLE III. Rats' Blood Urea-N after Ingestion of Human Blood.

| Supplement, % (W/V) | | | Avg urea-N, mg %, after | | |
|---|---|---|---|---|---|
| Iso-leucine | Methio-nine | ml/rat | 3 hr | 5 hr | 7 hr |
| | | 24 (4) | 24 (1) | 26 (1) | 29 (2) |
| | | 14 (6) | 20 (2) | 27 (2) | 26 (2) |
| 1.15 | .31 | 23 (4) | 22 (1) | 30 (1) | 27 (2) |
| 1.15 | .31 | 14 (6) | 22 (2) | 24 (2) | 22 (2) |
| | | 0(10) | 15 (3) | 13 (3) | 15 (4) |

No. of rats in parentheses.

*Results.* The results of these experiments confirm and extend the earlier report on inadequacy of human blood for the protein depleted adult male rat(13). Human blood and plasma and canine blood are deficient in both isoleucine and methionine when used as protein sources for depleted rats (Tables I and II). When fed to supply 120 mg N/rat/day, dog blood was as deficient in isoleucine as human blood and somewhat more deficient in methionine. Approximately 55 mg of L-isoleucine and 15 mg of DL-methionine/120 mg of blood-N adequately supplemented human blood for protein repletion in the rat (Table III). Using the average L-isoleucine and L-methionine content of human blood obtained by analyzing 3 different samples (Table IV), these figures approximate the reported repletion minima(22). As previously reported(13), the rats refused the non-supplemented blood after the first few days of repletion. As expected, nutritive value of human plasma was enhanced somewhat by addition of methionine and isoleucine(21). The improvement, however, was not as great as in the case of whole blood. Plasma also proved more readily acceptable than blood, confirming its better amino acid balance.

TABLE IV. Approximate Amino Acid Content of Blood, Plasma, and Fibrin Hydrolysate. (g/16.0 g N.)

| Amino acids | Human blood | Dog blood | Human plasma | Fibrin hydrol-ysate |
|---|---|---|---|---|
| Isoleucine | .80 | 1.49 | 2.26 | 6.15 |
| Leucine | 15.4 | 18.1 | 11.95 | 15.3 |
| Valine | 9.51 | 9.12 | 7.86 | 6.4 |
| Threonine | 4.86 | 5.36 | 4.01 | 6.4 |
| Methionine | 1.24 | .59 | 2.30 | 3.20 |
| Phenylalanine | 6.84 | 7.05 | 6.21 | 3.84 |
| Histidine | 6.42 | 5.19 | 3.21 | 2.44 |
| Arginine | 3.60 | 3.15 | 4.43 | 6.00 |
| Tryptophan | 1.34 | 1.06 | 1.61 | 1.23 |
| Glutamic acid | 9.13 | 8.25 | 14.47 | 5.16 |
| Aspartic acid | 5.40 | 5.57 | 5.22 | 2.36 |
| Tyrosine | 3.66 | 3.99 | 3.48 | 3.20 |
| Lysine | 9.20 | 9.76 | 10.11 | 9.1 |

Ingestion of 12-25 ml of citrated human blood increased blood urea-N in rats (Table IV). This agrees with the work of others with other species and other proteins(7,8,23). Supplementing the blood with 1.15 g of L-isoleucine and 0.31 g of DL-methionine/100 ml, however, did not reduce the rats' blood urea. Feeding dogs 300 mg N/kg/day as dog blood, or 150-375 mg N/kg/day as human blood, did not produce increased blood urea or NPN levels or negative nitrogen balance. Thus it would appear, either that the marked azotemia following ingestion of large amounts of blood is not related to amino acid imbalance, or the conditions in this experiment were not suited to demonstrate such an effect. When blood fortified with amino acids is given rapidly by stomach tube, the supplemental amino acids may be absorbed faster than those derived from the blood. This probably reduces the efficacy of the supplements. The rats fed supplemented blood twice daily by stomach tube (Table I), gained less than those allowed to drink the fortified blood. This suggests that the isoleucine and methionine might be more effective if given throughout the entire time interval in which the blood proteins are digested.

Analysis of blood and plasma for 13 amino acids revealed that human and dog blood differ principally in their content of isoleucine, leucine, and methionine (Table IV). Approximate amino acid content of the modified fibrin hydrolysate used is shown for comparison. Human blood contains more methionine, but less isoleucine and leucine than dog blood. These differences may be due chiefly to differences in amino acid composition of the hemoglobins(24). It is of interest that although dog blood contains twice as much isoleucine as human blood and two-thirds as much as human plasma, supplementing it with methionine did not increase the gains of rats (Table I). The excess of leucine in dog

blood may increase the isoleucine requirement. Thus, it has been shown that leucine increases the rat's isoleucine requirement(25) and that infusion of leucine into the blood of dogs increases urinary excretion of isoleucine as much as 140 times(26). Excess leucine reduced the rate of intestinal absorption of isoleucine by about 25%(27).

*Summary.* (1) These studies further evaluate whole blood as sources of essential amino acids for protein depleted adult male rats. Human and canine blood again were rejected and failed to support repletion. Human blood supported rapid repletion when supplemented with 55 mg L-isoleucine and 15 mg DL-methionine per 120 mg of blood-N. Less than half these levels added to plasma supported repletion. Plasma alone permitted an intermediate rate of repletion. (2) Feeding normal dogs a non-protein diet and 150-375 mg N/kg/day as lyophilized human or dog blood did not increase the blood urea-N or NPN or produce negative N balance. Force-feeding 12-25 ml of citrated human blood increased blood urea-N of fasting rats. Supplementing the blood with isoleucine and methionine, however, did not reduce the blood urea-N. (3) Analysis of human blood, human plasma, and dog blood for 13 amino acids is reported.

The authors wish to acknowledge the valuable assistance provided by B. R. Ryan, L. E. M. Crawford, and Eleanor Willerton.

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Received November 19, 1956. P.S.E.B.M., 1957, v94.

# Effects of Reserpine and Chlorpromazine in Prevention of Cerebral Edema and Reversible Cell Damage.* (23010)

PETER H. BULLE. (Introduced by Theodore Koppanyi)
*Department of Pharmacology, Schools of Medicine and Dentistry, Georgetown University, Washington, D.C.*

In an attempt to elucidate the mechanism which may cause or contribute to edema formation, the action of serotonin (5-hydroxytryptamine) on the brain was investigated. It is known that this substance is liberated or activated whenever thrombocytes disintegrate during clotting(1) and that it induces local edema upon subcutaneous injection(2). As a result of *in vivo* experiments it was found to act on the synaptic transmission(3). This last finding suggested that serotonin may have a neurotropic property through which it could theoretically contribute to the production of reversible hemiplegic symptoms. A series of tests was, therefore, carried out to establish the possible existence of such a relationship.

Should serotonin prove to be capable of causing cerebral edema as well as hemiplegic symptoms, it would then be logical to search for compounds counteracting these effects. Among drugs known to be inhibitory to certain actions of serotonin, reserpine and chlorpromazine were chosen. These two agents seemed particularly promising as reserpine was reported to prevent subcutaneous edema resulting from a challenging dose of egg-albumin in a heterogeneous animal(4) and chlorpromazine inhibited serotonin-induced subcutaneous edema(2).

*Methods.* 1) The edematous response of brain-tissue to serotonin was tested in albino mice. Groups of 40 mice, weighing approximately 25 g were used. Each experimental animal received 0.2 ml of a 0.4% solution of T-1824 (Evans' blue) in isotonic saline, intravenously. (This dye, which is bound to the plasma protein, stays within the vascular bed. Leakage of plasma into the tissue can thus be determined from the intense blue coloring.) Immediately upon each dye administration, one cerebral hemisphere of each experimental animal was injected with 0.05 ml of a 0.1% solution of serotonin in isotonic saline. Incidence of hemorrhage due to cerebral serotonin injection was not greater than that due to isotonic saline injection. Serotonin, however, induced edema, whereas saline did not. This indicates that under the experimental conditions serotonin did not increase the fragility of cerebral vessels. Twenty-four hours after injection, the test animals were killed by ether vapor inhalation and the intracranial contents carefully removed, frozen, and sliced by microtome technic. 2) Control studies were carried out with the same number and strain of mice, and animals treated in an identical manner, except that instead of serotonin-isotonic saline, Tyrode or Locke solution were injected intracerebrally. 3) The method for testing efficacy of reserpine and chlorpromazine in the prophylactic treatment of cerebral edema included intraperitoneal administration of varying amounts of the 2 drugs in isotonic saline solution to albino mice. Forty-five to 75 minutes later, each experimental animal received Evans' blue intravenously, immediately followed by intracerebral administration of serotonin. 4) The investigation of the role of serotonin in production of reversible hemiplegic symptoms was tested in 2 series of experiments: a) Four dogs received a unilateral internal-carotid injection of 3 mg/kg body weight of serotonin creatinine sulfate (of a 0.3% solution in isotonic saline). b) Another 4 dogs received unilateral internal-carotid injection of 0.003 $\mu$g/kg serotonin (a 0.003% solution in isotonic saline). 5) Alterations in the neurological serotonin response of the brain due to premedication with chlorpromazine and reserpine were tested in 2 series of experiments: a) Two dogs were

* The author gratefully acknowledges the research assistance given to him by Dr. E. C. Bulle and the technical assistance rendered by Mr. F. Migliorelli and Messrs. F. F. Cowan and D. T. Walz, graduate students of Georgetown University.

premedicated with 0.25 mg and 2 dogs with 1 mg/kg body weight chlorpromazine, intravenously, 2 hours prior to serotonin injection (route of administration and dosage identical to those in 4a). b) Another 2 dogs were premedicated with 0.0001 mg and 2 dogs with 1 mg/kg reserpine, intravenously, 3 hours prior to serotonin injection (route of administration and dosage identical as in 4b). 6) In order to establish whether the hemiplegic symptoms resulting from serotonin administration are due to cerebral edema or possibly to a neurotropic effect of that agent, experiments 4a), 5a) and 5b) were repeated with the only modification that each animal, immediately prior to serotonin administration, received an intravenous injection of 2 ml/kg of 0.4% solution of Evans' blue.† After approximately 1 hour, the dogs were killed by sodium pentobarbital injection, the brains removed and examined for dye diffusion or increase of moisture content in serotonin-injected hemispheres (by comparison of weight of the 2 hemispheres). 7.) In order to establish whether the hemiplegia-like symptoms are due to the neurotropic or the musculotropic property of serotonin, determinations of internal jugular blood-flow were carried out (bubble-flow meter).

*Results.* 1) Each of the cerebral hemispheres injected with serotonin contained one blue globule of 2 mm diameter, indicative of edema. 2) None of the hemispheres injected with physiological salt solutions showed edema formation, with the exception of those which had trauma-induced hemorrhages. In other words, it could be established that exogenous serotonin produces cerebral edema, whereas physiological salt solutions do not. 3) Examination of the brains of mice, pretreated with reserpine or chlorpromazine, showed that reserpine in concentration of $1x10^{-5}$ $\mu$g/kg body weight in 0.1 ml saline inhibited the serotonin-induced edematous response of brain tissue in all test animals. With the use of $1x10^{-7}$ $\mu$g/kg in 0.1 ml of isotonic saline, 50% of the experimental animals were still protected against the edema-producing property of serotonin (Table I). In the case of chlorpromazine, 0.1 $\mu$g/kg body weight was needed to protect all animals, and 0.01 $\mu$g/kg to prevent edema formation in 50% (Table II). 4a) Serotonin in very large dosage (3 mg/kg) induced in all 4 animals the following immediate reactions: ipsilateral ptosis, mydriasis and slight facial paralysis, together with contralateral flaccid paralysis of all musculature innervated by spinal nerves. These hemiplegia-like signs were of a very transient nature, lasting less than 5 minutes, during which time the dogs defecated and urinated. 4b) Upon serotonin administration in very small dosage (0.003 $\mu$g/kg), all 4 animals showed ipsilateral miosis and spastic facialis paralysis along with contralateral spastic paralyses. These hemiplegia-like signs were evident for less than 5 minutes. (To exclude errors from individual variations, the same dogs, after full neuro-

† All surgery was performed on animals under ethyl chloride anesthesia.

TABLE I. Effects of Reserpine on Serotonin-Induced Edema in Mice.

Reserpine — Intraper. admin. Dosages shown in tabulation.

Evans' blue— Intrav. admin. (tail vein, 0.2 ml of 0.4% in isotonic saline) 2 hr following reserpine.

Serotonin — Immediately following Evans' blue, 50 $\mu$g total intracer. admin. in 0.05 ml of isotonic saline.

| No. of animals surviving* | Reserpine dosage ($\mu$g/kg) | With hemorrhage | | No hemorrhage | |
|---|---|---|---|---|---|
| | | No diffusion | Diffusion | No diffusion | Diffusion |
| 10 | 1000 | 2 | 1 | 7 | 0 |
| 10 | 100 | 2 | 2 | 6 | 0 |
| 10 | 10 | 3 | 1 | 6 | 0 |
| 10 | 1 | 2 | 2 | 6 | 0 |
| 15 | .1 | 0 | 4 | 11 | 0 |
| 12 | .01 | 0 | 4 | 8 | 0 |
| 12 | .001 | 0 | 5 | 7 | 0 |
| 16 | .0001 | 0 | 7 | 9 | 0 |
| 13 | .00001 | 0 | 4 | 9 | 0 |
| 12 | .000001 1:million | 0 | 3 | 8 | 1 |
| 13 | .0000001 1:10 mil. | 0 | 3 | 6 | 4 |
| 13 | .00000001 1:100 mil. | 0 | 4 | 3 | 6 |
| 12 | .000000001 1:1000 mil. | 0 | 2 | 2 | 8 |

* 40 mice used at each dose level.

TABLE II. Effect of chlorpromazine on Serotonin-Induced Edema in Mice.

Chlorpromazine — Intraper. admin. Dosages shown in tabulation.

Evans' blue — Intrav. admin. (tail vein, 0.2 ml of 0.4% in isotonic saline) 2 hr following chlorpromazine.

Serotonin — Immediately following Evans' blue, 50 μg total intracer. admin. in 0.05 ml of isotonic saline.

| No. of animals surviving* | Chlorpromazine dosage (μg/kg) | With hemorrhage | | No hemorrhage | |
|---|---|---|---|---|---|
| | | No diffusion | Diffusion | No diffusion | Diffusion |
| 13 | 10,000 | 0 | 3 | 10 | 0 |
| 21 | 1,000 | 2 | 5 | 14 | 0 |
| 16 | 100 | 1 | 3 | 12 | 0 |
| 18 | 10 | 2 | 5 | 11 | 0 |
| 15 | 1 | 3 | 2 | 10 | 0 |
| 22 | .1 | 4 | 7 | 11 | 0 |
| 23 | .01 | 3 | 10 | 5 | 5 |
| 24 | .001 | 7 | 4 | 4 | 9 |
| 28 | .0001 | 2 | 9 | 1 | 16 |
| 19 | .00001 | 0 | 8 | 0 | 11 |

* 40 mice used at each dose level.

Approximately 30% of the animals, which received intracer. inj. (Tables I and II) died within 20 min. due to extensive hemorrhages. Approximately 30% of the surviving animals showed minute hemorrhages in the area of needle insertion, in histological examinations of serial sections. In the "no-hemorrhage" category of animals, the absence of hemorrhage was determined by histological examination of serial sections.

logical recovery, were subjected to a second serotonin injection into the same artery with identical amounts and concentrations as used in 4a. The resultant neurological findings were exactly the same as observed in 4a. In other words, the spastic paralysis from low dosage was converted into a flaccid paralysis from high dosage.) 5a) The response of all chlorpromazine premedicated animals to serotonin administration consisted in a marked amplification of the signs and their duration observed in control animals in 4a. In addition, the ipsilateral musculature of the face in some instances showed fasciculation. All these symptoms were evident for more than one hour. In analogous experiments, chlorpromazine did not noticeably alter the stimulating effect of serotonin administered in small doses. 5b) The result of reserpine premedication in all 4 animals was a very slight ipsilateral pupillary dilatation (during the large dose injection period only, altogether lasting less than 3 seconds). The dogs did not show any signs of ipsilateral spastic or flaccid facialis paralyses, nor did they have contralateral spastic, respectively-flaccid paralyses. 6) Dye administration did not alter the serotonin-induced hemiplegic symptoms, nor could gross edema formation and increase of weight be detected in the serotonin-injected hemispheres of any animal. 7) Internal jugular blood-flow was not altered due to injection of serotonin into the internal-carotid artery, thereby suggesting that the neurotropic effect of serotonin is responsible for the development of hemiplegic symptoms.

*Discussion.* Our findings raise the question as to whether or not a portion of the post-apoplectic edema may be due to serotonin release from disintegrating thrombocytes. A possible explanation for the mechanism of such edema formation may be sought in the known positive musculotropic effects of serotonin. This agent, entering the interstitial spaces of the brain, may thus be expected to cause local vasoconstriction, which in turn leads to ischemia and an increase of the cell permeability. Concomittant neurological changes in the contralateral musculature of extremities indicate that the facial paralyses were due to brain responses rather than to spilling of injection fluid. Moreover, the internal carotid artery of the dog has no branches supplying facial musculature. Reserpine and chlorpromazine were found to prevent the cerebral edema-inducing effect of serotonin. It appeared, however, that cerebral edema in itself does not produce the reversible hemiplegic symptoms, but that it is the neurotropic action of serotonin which may be held responsible for these symptoms. Edema *per se* may, of course, prolong and thus aggravate the damage to the brain-cells involved. Chlorpromazine premedication potentiates the neurotropic action of large doses of serotonin; in other words, it aggravates flaccid paralyses. It does not influence the spastic paralyses as caused by serotonin in small dosage. Reserpine premedication inhibits the neurotropic action of serotonin, regardless of whether the latter agent was ad-

ministered in stimulating or depressing dosage. It was not possible to test the therapeutic efficacy of reserpine after serotonin administration, as the neurological symptoms induced by intra-arterially injected serotonin were of too short duration.

*Summary.* 1) Serotonin, administered into brain-tissue via an extravascular route, induces edema, possibly due to its musculotropic property. 2) Serotonin was confirmed to have a neurotropic action which appeared to be responsible for the reversible hemiplegic symptoms. 3) The neurotropic effect of serotonin is biphasic, *i.e.* when administered in low dosage via internal-carotid artery, it causes transient spastic paralyses. In high dosage, it causes flaccid paralyses without inducing cerebral edema. 4) Chlorpromazine and reserpine both block the edema-inducing action of serotonin. Reserpine is, however, effective in a much lower dosage. 5) Chlorpromazine potentiates the neurotropic depressant effect of serotonin, but apparently does not alter its stimulating action. 6) Reserpine inhibits the neurotropic effect of serotonin. This holds true for the spastic as well as for the flaccid symptoms. 7) Therapeutic considerations suggest that reserpine may be specifically indicated in prophylactic treatment of apoplexy.

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Received November 26, 1956. P.S.E.B.M., 1957, v94.

## Japanese B Encephalitis Virus in Tissue Culture.* (23011)

Robert W. McCollum and John F. Foley. (Introduced by J. L. Melnick)
*Section of Preventive Medicine, Yale University School of Medicine, New Haven, Conn.*

The virus of Japanese B encephalitis (JBE) grows readily in a variety of tissue culture systems. Kawakita(1) successfully propagated JBE virus serially in Maitland-type cultures of minced chick embryo brain, and in minced whole chick embryo(2). Scherer and Syverton(3) reported 13 serial passages of JBE virus in HeLa cell cultures. Cytopathogenic effect (CPE) was noted only with the first 4 passages although continued virus proliferation was evidenced by mouse pathogenicity of subsequent tissue culture passages. Mason reported growth of 2 strains of JBE virus in HeLa cell cultures with CPE noted only in 13th and 41st passages of one strain and only in 32nd passage of the other. In general, however, consistent and reproducible CPE has not been observed. The present report is concerned with the propagation of JBE virus in several tissue culture systems, primarily in the cell line currently known as Detroit-6 (originally isolated by Berman, Stulberg and Ruddle(4) from human bone marrow), the only type of cells which has shown consistent CPE in our experience.

* Work sponsored by Com. on Viral Infections, Armed Forces Epidemiological Board, and supported by Office of Surgeon General, Department of Army.

*Materials and methods. Viruses: a) Japanese B. encephalitis.* Strain $M_1311$ was received as the seventh mouse brain passage of a virus isolated from a pool of *Culex tritaeniorrhynchus* mosquitoes. These were trapped near Tokyo, Japan in 1951 by members of the 406th Medical General Laboratory (U.S. Army). Strain $M_3$2204 B was received as the third mouse brain passage of a similar isolation made in 1953. Both were kindly supplied by Major Edward L. Buescher, Walter Reed Army Institute for Research. Both strains were passed one time in suckling Swiss albino mice for stock suspensions, used to initiate tissue culture passages. *b) West Nile.* Strain EV 101 was supplied as third mouse brain passage by Dr. Joseph L.

Melnick. *Detroit-6 cell cultures*: The original supply was kindly furnished by Drs. I. W. McLean and W. A. Rightsel of Parke, Davis and Co., Detroit, Mich. Stock cultures in 3 oz. pharmacy bottles were maintained in 15% pooled human serum in Eagle's synthetic medium(5). When a confluent sheet had formed (usually 5 to 7 days after seeding and one medium change) the cells were scraped off with a rubber policeman into 20 ml of fresh medium. (For some cultures 40% pooled human serum in Hanks' balanced salt solution was used.) A dispersion consisting predominantly of single cells was obtained by passing this suspension repeatedly through a 22 gauge needle attached to 2 ml automatic Cornwall syringe. The suspension was diluted with growth medium to a concentration of 200,000 cells/ml. Tubes receiving 0.5 ml of this suspension were incubated at slight slant at 36-37°C. They were fed the next day with 0.5 ml of fresh growth medium. Solid sheets of cells were present after 3 to 5 days. Media were usually changed 24 hours prior to inoculation. The cultures were washed 2 to 3 times with Hanks' BSS immediately prior to introduction of 0.9 or 1.8 ml of maintenance solution (10% normal horse serum, 10% tryptose phosphate solution, and 80% mixture 199) and an inoculum of 0.1 or 0.2 ml of a virus or control suspension in the same medium. Cultures were observed daily for 8 to 12 days and fluids were changed approximately every 4 days. *Other tissue culture systems*: 1) *Chick embryo fibroblast cultures* were prepared by 2 methods: a) implantation in chicken plasma clot of skin-muscle fragments from 9-day-old chick embryos; b) direct growth on glass of trypsinized cells from minced torsos of 9-day-old chick embryos. 2) HeLa cells were obtained from Microbiological Associates ready for inoculation. 3) Monkey kidney epithelial cells were prepared by the method of Rappaport(6). *Assay of tissue culture fluids for virus content*: The same method was used for all cell lines. Eight to 10 g, weanling Swiss albino mice were inoculated by intracerebral route with 0.03 ml of serial 10-fold dilutions of tissue culture fluids (diluted in same medium as that used for maintenance of the cell line). Specificity of animal deaths was ascertained at various tissue culture passage levels by use of hyperimmune guinea pig serum. *Tissue culture neutralization tests*: Virus (tissue culture fluids) and serum dilutions were prepared in ice bath (2-3°C) so that in the final mixture a volume of 0.1 or 0.2 ml contained approximately 100 or 1000 $TCD_{50}$ of virus, and serum in a final dilution of 1:10 or 1:100. These mixtures were then incubated at 37°C for one hour prior to inoculation of the cultures. Simultaneous virus titrations and serum controls were included. *Preservation of tissue culture passage fluids*: Aliquots of fluid pools from each passage level were shell frozen in glass sealed ampoules in a $CO_2$-alcohol bath and stored at –70°C in a $CO_2$ chest.

*Results*. Evidence of continued virus proliferation was obtained with 10 or more serial passages of both strains ($M_1$311 and $M_3$2204 B) of JBE virus in each of the cell lines studied. Maximal virus titers in the range of $10^{4.0}$ to $10^{7.0}$ mouse $LD_{50}$/ml were observed in chick embryo fibroblast, HeLa cell and monkey kidney epithelium cultures, but no specific, consistent or reproducible CPE was observed. Such an effect was noted in the first 15 continuous passages of strain $M_3$2204 B in the plasma-clot cultures of chick

TABLE I. Comparison of Serum Neutralization Tests in Mice and Tissue Cultures (Dt-6 Cells).

| Serum (1:10 final dilution when mixed with virus dilutions) | | JBE virus—strain $M_1$311 | |
|---|---|---|---|
| | | 8th passage mouse brain* LNI‡ | 12th passage T.C. fluid† LNI‡ |
| Guinea pig | Normal pool | .0 | .0 |
| | Immune " | 3.2 | 2.8 |
| Human A | Pre-vacc. | .0 | .3 |
| | Post " | 1.8 | 1.8 |
| B§ | Pre " | 1.6 | 1.3 |
| | Post " | 3.4 | 2.7 |

* Titer in 8-10 g mice (intracer.) = $10^{-7.4}$.

† Representing a total accumulative dilution of $10^{-27}$ of 8th mouse brain passage. Titer in Dt-6 cells (by CPE) = $10^{-5.3}$.

‡ LNI = Log Neutralization Index calculated on the basis of 5 mice or 3-4 tube cultures inoculated per virus dilution-serum mixture.

§ Prior to vaccination this individual had acquired antibodies through inapparent infection during Army duty in Japan and Korea.

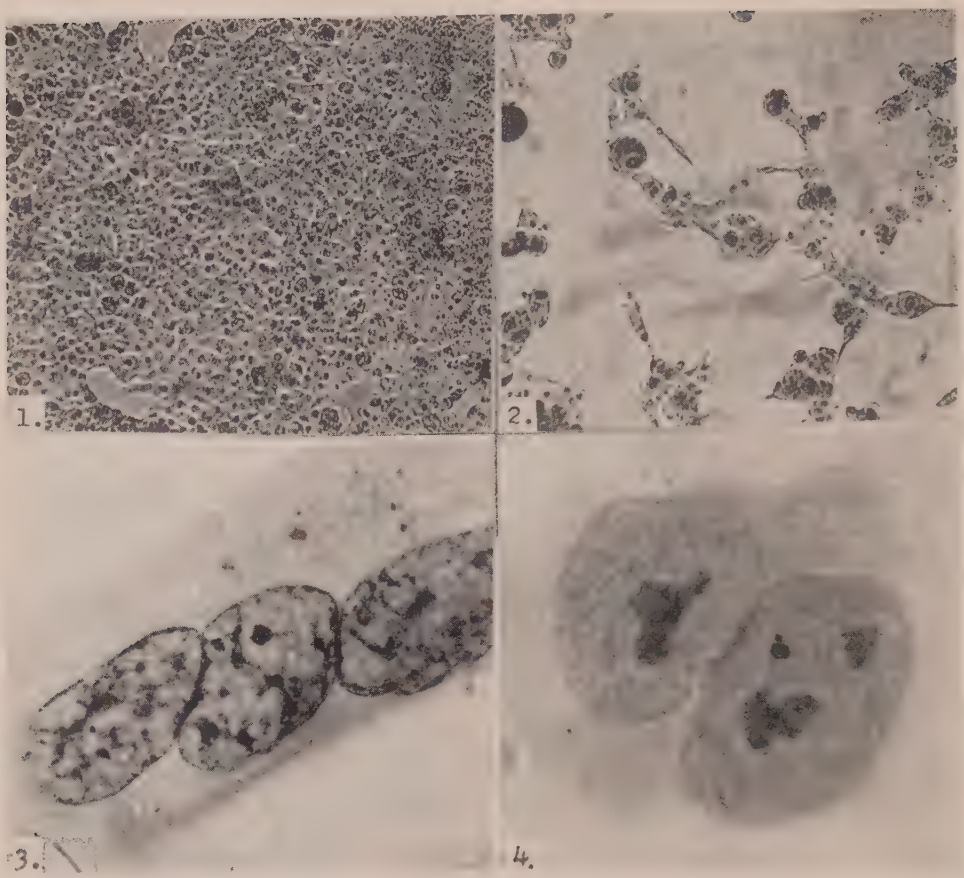



FIG. 1. Dt-6 cell culture showing appearance of uninfected cells 10 days after inoculation of undiluted control fluid pool from previous passage. Unstained preparation, approx. X 80.

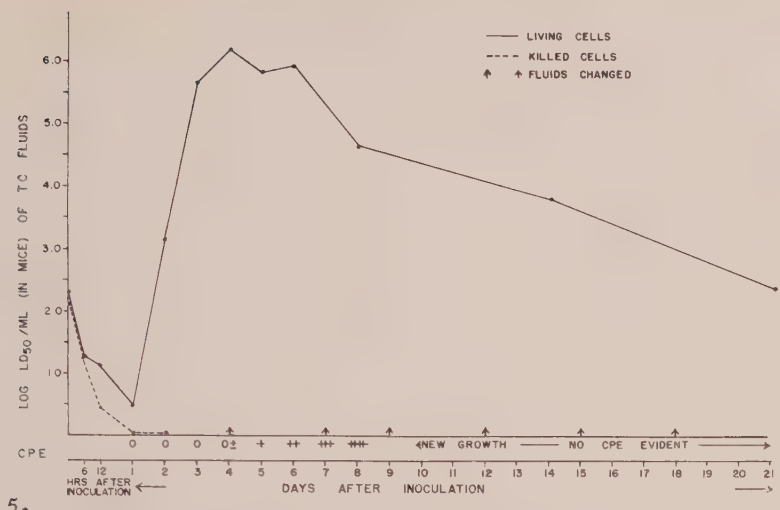
FIG. 2. Dt-6 cell culture showing advance CPE 10 days after inoculation of 32,000 $TCD_{50}$ of 9th passage JBE virus, strain $M_1311$. Onset of CPE was on 8th day. Unstained preparation, approx. X 80.

FIG. 3. Dt-6 cells 6 days after inoculation (10,000 $TCD_{50}$ JBE virus, strain $M_1311$) showing cytoplasmic contraction, thickening of nuclear membrane, clumping of chromatin and fragmentation of nucleoli. Such changes are most obvious in the larger multinucleated cells which are scattered throughout the cultures. H and E stained coverslip preparation, approx. X 800.

FIG. 4. Dt-6 cells 6 days after inoculation of control fluid from previous passage. Cytoplasmic boundaries are not seen. Chromatin is finely granular. Nucleoli are irregular but intact. H and E stained coverslip preparation, approx. X 800.

embryo fibroblasts. However, it could not be reproduced with the frozen tissue culture passage materials and its disappearance remains unexplained.

A cytopathogenic effect was observed, however, with each passage of both strains in Detroit-6 cells and this effect has been reproduced with frozen materials from representative passages at all levels. Through 16 passages of strain $M_1311$ the cumulative total dilution of the original mouse brain inoculum was $10^{-43}$. The titer of the 16th passage fluid was $10^{-6.0}$/ml in mice and $10^{-5.5}$/ml in tissue culture (based on CPE). In general, comparative titrations in mice and tissue cultures have given essentially equal results. Through 7 passages of strain $M_32204$ B the virus titers, as determined by CPE or mouse infectivity, have been similar. Neutralization of the CPE produced by JBE ($M_1311$) virus in Detroit-6 cells has been demonstrated (Table I) by use of hyperimmune guinea pig serum and human immune serum (both post-infection and post-vaccination).




5.

FIG. 5. Growth curve of JBE virus, strain $M_3311$, 12th Dt-6 tissue culture passage. Titers are expressed as mouse $LD_{50}$/ml of pooled fluids from 4 tissue culture tubes. Degenerated cell sheet had completely recovered by 14th day. No further CPE was observed even though virus proliferation continued.

Cytopathogenicity usually becomes apparent on 5th day after inoculation, but may appear as early as the fourth day or as late as the eighth day, depending to some extent on dilution of the inoculum. The earliest evidence of CPE consists of small focal areas of heaped-up, rounded cells. Shortly thereafter the cellular sheet in these areas begins to retract and scattered, large, swollen cells may be noted. Within several hours retraction has increased so that the sheets exhibit a "Swiss cheese" appearance. During the next 24 to 48 hours large numbers of cells become detached. Degeneration proceeds rapidly but is never 100% complete. Fig. 1 and 2 illustrate respectively a control culture and an infected culture in an advanced state of degeneration.

Hematoxylin and eosin stains of JBE infected Detroit-6 cells show changes confined primarily to the nuclei though cytoplasmic contraction is apparent. Nuclear changes consist of washing out of the fine chromatin material, clumping and thickening of chromatin toward periphery of the nucleus, thickening of the nuclear membrane, fragmentation of the nucleoli, and the appearance of variable sized eosinophilic inclusions. There is rather marked variation in the size of cells and nuclei within a given culture and these changes are most striking in the larger cells which are often multinucleated. Fig. 3 shows such nuclei demonstrating these changes as compared, in Fig. 4, with the appearance of nuclei of similar size in an uninfected control culture of the same age.

Limited growth studies (Fig. 5), determined by measurements of extracellular virus, indicate that there is an initial drop in titer during the first 24 hours. For the first 6 hours this seems to parallel the drop in titer observed in virus inoculated cultures of cells killed by repeated freezing and thawing. Virus proliferation (or release) is maximal on the fourth day, one day prior to the first definite evidence of CPE. A relative plateau is maintained for 2 days after which the titer falls gradually. Cultures which have exhibited maximal (but not 100%) degeneration show continued cellular proliferation if the fluids are changed frequently. One such group of cultures, kept for 21 days (with fluids changed 6 times), yielded fluid with a virus titer of $10^{2.3}$ mouse LD50/ml, an amount equivalent to the original inoculum.

Limited attempts to utilize the fluids from various tissue culture systems as complement-fixing and hemagglutinating antigens have been essentially unsuccessful.

West Nile (WN) virus (Egypt 101)

through 6 serial passages in Detroit-6 cells gave a picture identical to that observed with JBE virus. In general, the onset of CPE was noted one to 2 days later than with JBE virus, but degeneration progressed in the same manner.

*Discussion.* Although these studies confirm previous observations(1-5) that JBE virus grows readily in a variety of tissue culture cell lines, CPE has previously been a rare indicator of virus production and its appearance has been irregular. Detroit-6 cells appear to be unique among cell systems used thus far, in that CPE has served as a consistent indicator of virus proliferation and infectivity. The fact that degeneration has never been total and cellular and viral proliferation will continue for prolonged periods under proper conditions suggests the possibilities that: 1) some cells undergo a mutation or change rendering them unsusceptible to CPE while leaving them susceptible to infection; or 2) there are actually 2 (or more) different cell types in the cultures capable of supporting virus multiplication, but only one subject to degeneration in the process. In fact, careful microscopic examination of our Detroit-6 cell cultures has revealed the presence of what appear to be several cell types. The cells which degenerate, however, would appear to be more efficient virus producers since virus titers are highest prior to their gross degeneration (see growth curve). Fluids harvested on 3rd to 6th day (0-2+ degeneration) have consistently yielded higher titers than those tested at later times even when renewed growth has produced an almost complete sheet of cells.

The failure to obtain higher virus infectivity titers in Detroit-6 cells as well as in other cell systems used, is probably due to the marked thermolability of JBE virus. Tissue culture fluids containing over 5 logs of virus/ml (as measured by mouse infectivity immediately after harvesting) had dropped to less than 1 log/ml after 12 hours incubation at 37°C. and no detectable mouse or tissue culture infectivity after 24 hours. Since the cultures are maintained at 36-37°C, there would appear to be a high ratio of inactive to active virus in the fluids at any time beyond the first few hours after beginning of release of virus from the cells. It has been observed repeatedly that CPE becomes apparent from one to 2 days earlier in cultures receiving 100 to 1000 $TCD_{50}$ than in cultures inoculated with larger doses. That this delay is probably due to interference was demonstrated by inoculating cultures with large amounts of inactive virus (kept at 37° for 24 hours) 6 hours prior to the addition of active virus. The CPE in these tubes appeared from 2 to 3 days later than in controls inoculated with the active virus only.

The fact that West Nile virus, another member of the serologically related B group of the arthropod-borne viruses, produces a CPE in Detroit-6 cells similar to that produced by JBE virus suggests the possibility that this cell line might be susceptible to other members of the same group.

*Summary.* Two strains of Japanese B encephalitis virus have been serially propagated in tissue cultures of chick embryo fibroblasts (both plasma-clot explants and trypsinized cells), monkey kidney epithelium, HeLa cells, and Detroit-6 cells. Cytopathogenicity, which was consistent and reproducible, was observed only with the Detroit-6 cells. Maximal virus growth and release was observed 2 days before CPE became pronounced. This effect was readily neutralized by JBE immune serum. A similar CPE has been observed in Detroit-6 cells infected with the Egypt 101 strain of West Nile virus.

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Received November 28, 1956. P.S.E.B.M., 1957, v94.

# Influence of Respiratory Acidosis on ECG and Pressor Responses to Epinephrine, Norepinephrine and Metaraminol.* (23012)

Dudley B. Houle, Max H. Weil, E. B. Brown, Jr. and Gilbert S. Campbell
*Departments of Surgery & Physiology, University of Minnesota Medical School, Minneapolis.*

Mines(1), reported that perfusion of an isolated frog heart with acid lengthened the A-V interval and led to heart block and that "perfusion with an alkaline solution quickly removed the block and then steadily reduces the A-V interval." Andrus and Carter(2) found that rhythm and conduction time in isolated dog's and terrapin's hearts could be correlated with pH of the perfusate. When an acid perfusate was used, rate slowed and conduction time lengthened. Opposite effects were observed when an alkaline perfusate was used. Andrus(3) noted that the stimulating effect of adrenalin and tyramine upon isolated auricles of the rabbit was enhanced by pH of 7.8 and diminished by pH of 7.0. Burget and Visscher(4) reported the relationship between adrenalin effect and blood pH in pithed cats. They found progressively greater adrenalin effects with increasing blood pH up to 8.0.

*Methods.* Dogs were anesthetized with intravenous 5% sodium pentobarbital (30 mg/kg). Gas mixtures were administered through a cuffed endotracheal tube. Blood pressures were recorded through an indwelling femoral artery catheter connected to a pressure transducer and thence to a 2 channel direct writing oscillograph. Mean arterial blood pressures were recorded by use of an electrical integrating circuit in the oscillograph. In the first phase of this investigation electrocardiograms were recorded on the second channel of the oscillograph. Arterial blood samples were drawn through the femoral artery cannula and blood pH determinations were made on these samples. Blood pH was determined at 38°C with a specially constructed glass electrode pH meter(5). Five animals were subjected to respiratory acidosis by inhalation of 30% $CO_2$-70% $O_2$ gas mixtures. Sympathomimetic agents were administered intravenously by a femoral vein catheter. Doses and agents used were: epinephrine 2.7 $\gamma$/kg, norepinephrine 2 $\gamma$/kg, and metaraminol 73 $\gamma$/kg. These doses were calculated to produce approximately the same moderate elevation of mean arterial pressure in the intact dog with each of the drugs. Ten dogs were subjected to complete cardiac by-pass using a pump homologous lung oxygenator in the extra corporeal circuit(6). The roots of the aorta and pulmonary artery were clamped and the animals were maintained on total by-pass as long as 2 hours. The homologous lung was ventilated with 30% $CO_2$-70% $O_2$ for 15 minutes at 18-22 respirations per minute and at a pressure of 16-18 cm of water. Blood flow was regulated to maintain a mean systemic arterial pressure of 75 to 100 mm Hg. Following 15 minutes of equilibration on $CO_2$, the responses to intra-arterial epinephrine, norepinephrine and metaraminol were recorded. Doses used were: epinephrine 1.3 $\gamma$/kg, norepinephrine 1.0 $\gamma$/kg, and metaraminol 22 $\gamma$/kg. As soon as the animal's response to the 3 pressor agents was completed, an arterial blood pH was determined. The ventilating gas was then changed to 100% oxygen and the homologous lung ventilated with this gas for 15 minutes to lower the $CO_2$ content of blood and return the pH to a normal range. Following this period of equilibration, the same drugs at the same dosages were again administered and the pressor responses recorded. After the response to the last of the drugs was complete, the arterial blood pH was again determined.

*Results.* In the 5 intact dogs subjected to respiratory acidosis, average blood pH was 6.76 as compared with the average control pH of 7.34 while breathing room air. All sympathomimetic agents given in amounts indicated above produced cardiac irregularities in dogs at control pH. These arrhythmias

* This investigation was supported by Research Grant from N. Heart Inst., of U. S. Public Health Service and Minnesota Heart Assn.

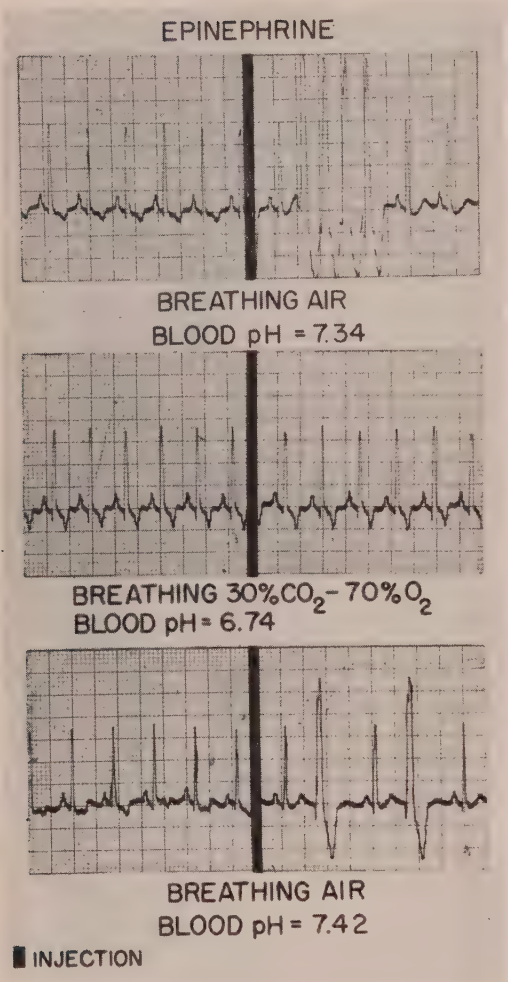


FIG. 1.

were ventricular extrasystoles originating from different foci, with frequent runs of ventricular tachycardia. In some tracings, changes in atrial conduction, with changing atrial pacemaker or nodal rhythm, were also observed. The ECG abnormalities were minimal or absent when identical amounts of these pressor agents were administered to acidotic animals (Fig. 1, 2, 3). As a rule, pressor responses in the presence of respiratory acidosis were reduced when compared with those responses obtained in the control state, although in some instances the differences were small (Table I).

In the 10 dogs subjected to total cardiac by-pass, mean arterial blood pH was 6.67 while the homologous lung was being ventilated with 30% $CO_2$-70% $O_2$. Blood pH rose to 7.41 after 15 minutes of ventilation of the homologous lung with 100% $O_2$. Average increases in mean arterial blood pressure to epinephrine, norepinephrine, and metaraminol during respiratory acidosis were 14, 16 and 23 mmHg respectively; at normal blood pH, mean pressor responses to these agents were 36, 26, and 38 mmHg respectively; (Table II). Statistical analysis of differences in pressor responses during acidosis and at normal blood pH reveals a P value of less than 0.001 for all 3 agents. For dosages employed, epinephrine and norepinephrine gave responses which

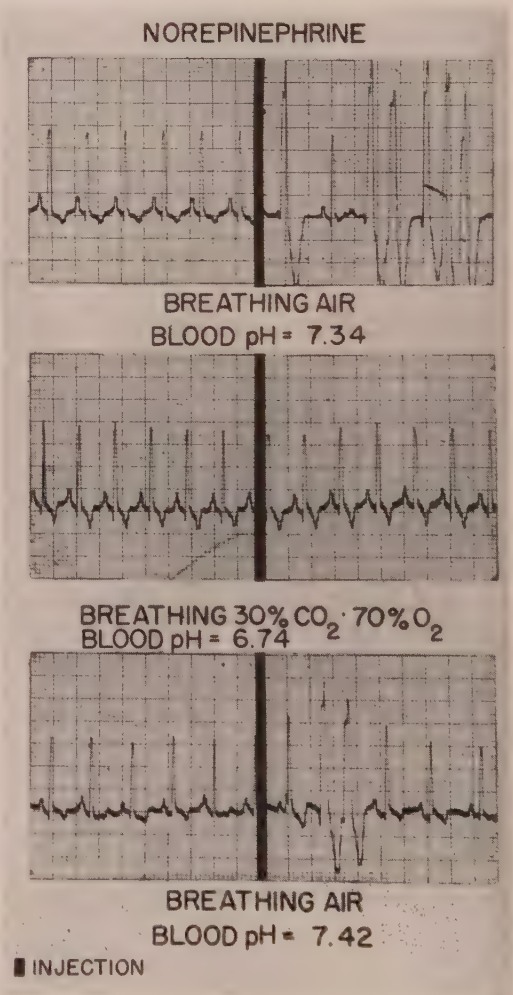


FIG. 2.

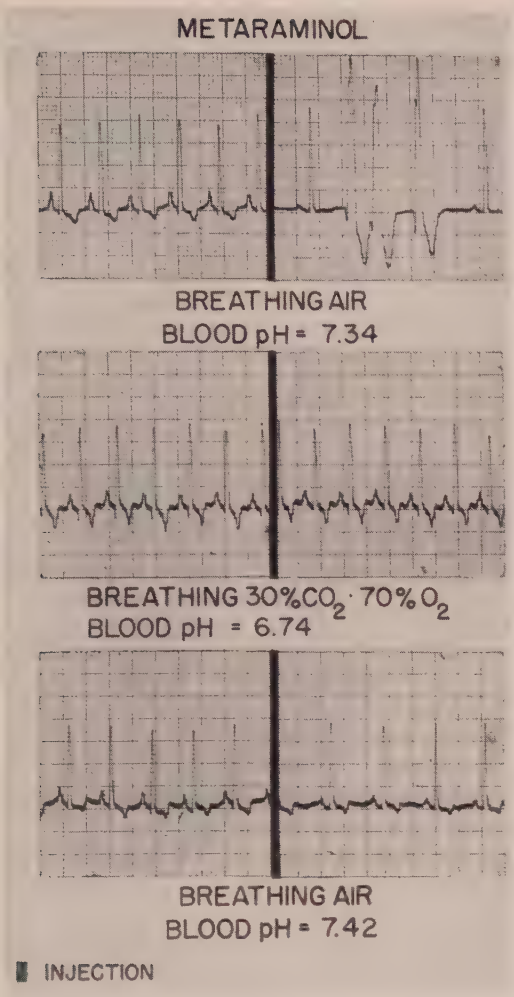


FIG. 3.

were comparable as to intensity and duration. Metaraminol gave a greater response than did the other agents and the duration of the pressor effect was more prolonged.

*Discussion.* In intact animals the differences in response during respiratory acidosis and under normal pH conditions were not uniformly large. This is particularly evident when acidotic and post acidotic responses in the intact dog are compared. The marked cardiac irregularities produced by these agents under normal blood pH probably masked the pressor response. It is also possible that the degree of bradycardia produced, via the parasympathetic pressoreceptor pathways, during acidosis may have affected the response in intact dogs.

In those experiments in which the heart was excluded from the circulation of the animals, and therefore did not play a role in the responses obtained, the agents were perfused only through the extra cardiac vascular system and a more accurate measurement of their effect on peripheral vascular resistance could be recorded. Obviously, these peripheral vascular resistance changes could be mediated through the central nervous system or through direct action on peripheral vessels. The experiments reported here do not separate these two possible sites of action of these drugs. The method of total cardiac bypass employed (the pump homologous lung oxygenator) serves as a useful experimental tool in separating cardiac and extracardiac effects of pharmacologic agents. Purposefully the control portion of the experiment, in which the animal's pH was near normal, was performed after the animal had been subjected to a period of respiratory acidosis, and consequently deterioration in the preparation would decrease the responses at normal pH rather than the responses obtained under conditions of lowered blood pH.

At the present time we are unable to interpret the mechanism by which severe respiratory acidosis decreases response to pressor

TABLE I. Pressor Responses before, during and after Respiratory Acidosis in Dogs.

| | Pre-acidosis | | Respiratory acidosis | | Post-acidosis | |
|---|---|---|---|---|---|---|
| | Pre-inj. B.P. (mm Hg) | Post-inj. B.P. incr. (%) | Pre-inj. B.P. (mm Hg) | Post-inj. B.P. incr. (%) | Pre-inj. B.P. (mm Hg) | Post-inj. B.P. incr. (%) |
| Epinephrine | 139 | 36 | 133 | 18 | 117 | 38 |
| Norepinephrine | 131 | 57 | 133 | 29 | 122 | 34 |
| Aramine | 122 | 59 | 142 | 21 | 128 | 28 |
| | pH = 7.34* | | pH = 6.76* | | pH = 7.27* | |

* Avg values for 5 dogs.

TABLE II. Pressor Responses during and after Respiratory Acidosis in Dogs during Total By-Pass.

| | Respiratory acidosis | | Post-acidosis | |
|---|---|---|---|---|
| | Pre-inj. B.P. (mm Hg) | Post-inj. B.P. incr. (mm Hg) | Pre-inj. B.P. (mm Hg) | Post-inj. B.P. incr. (mm Hg) |
| Epinephrine | 78 ± 3.5* | 14 ± 3.4 | 89 ± 5.6 | 36 ± 6.6 |
| Norepinephrine | 80 ± 2.3 | 16 ± 2.5 | 88 ± 6.8 | 26 ± 3.4 |
| Aramine | 84 ± 2.7 | 23 ± 2.7 | 92 ± 6.5 | 38 ± 4.7 |
| | pH = 6.67† | | pH = 7.41† | |

* ± stand. error of the mean. † Avg values for 10 dogs.

drugs. It is possible that changes in some component of plasma other than $H^+$, $CO_2$, or $HCO_3^-$ are responsible. It seems unlikely that changes in plasma potassium are responsible for the observed effects since plasma potassium changes very little in 15 minutes with 30% $CO_2$ breathing in the dog(7).

Page and Olmsted(8) reported that dogs subjected to respiratory acidosis (30% $CO_2$) were refractory to pressor drugs, whereas metabolic acidosis (0.1 normal hydrochloric acid intravenously) in dogs did not produce a depressed response to pressor agents. Studies are now in progress comparing pressor responses in dogs subjected to total cardiac by-pass at normal blood pH levels and during metabolic acidosis.

*Summary and conclusions.* 1. Sympathomimetic agents were administered to intact dogs under conditions of respiratory acidosis and at normal blood pH. Arterial pressure responses and electrocardiograms were obtained. In the presence of respiratory acidosis, the pressor responses were somewhat diminished and ECG abnormalities were absent or minimal as compared to the control state. 2. The same agents were administered to dogs subjected to total cardiac by-pass. Pressor responses under conditions of respiratory acidosis were uniformly much less in these animals than responses subsequently obtained in the same animals in which the arterial blood pH was at or near normal values.

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Received November 30, 1956. P.S.E.B.M., 1957, v94.

# Effect of Ablation of Neocortex on Ability of Pituitary to Secrete Thyrotropin in the Rat.* (23013)

MONTE A. GREER† AND HERBERT F. SHULL. (Introduced by R. L. Swank)
*Radioisotope Service, Vet. Admin. Hospital, Long Beach, Calif. and Department of Medicine, UCLA School of Medicine.*

It has been shown that lesions in the anterior hypothalamus will interfere with secretion of thyrotropin by the pituitary(1-4). Since it has not yet been ascertained whether the hypothalamus exerts a primary role in controlling thyrotropin secretion or merely transmits influences from higher centers, the present investigation was undertaken. Originally, it was planned to study thalamic preparations, but it was not possible to keep such animals alive more than one week in spite of forced-feeding and daily antibiotics. Therefore the operation was limited to removal of the neocortex. Such animals had a reasonable survival and permitted evaluation of the goitrogenic response to propylthiouracil administration. The size of the goiter produced was considered an index of the ability of such animals to secrete thyrotropin in response to maximal stimulus.

*Materials and methods.* Female Holtzman rats of the same age, weighing approximately 180-200 g were used as both control and experimental subjects. Removal of the neocortex was accomplished by exposing dorsum of skull through a longitudinal incision, scraping away the galea, and coagulating the underlying vascular tissue by application of electrocautery to the skull. A dental drill was then used to perforate the skull in several places along the border of muscle insertions and the piece of bone was removed with the aid of small bone cutters. This exposed most of the dorsum of the brain with very little bleeding. The neocortex was then removed by suction through a fine glass pipette under direct vision. The ablated cortical tissue was replaced by small pieces of oxycellulose and the skin sutured over the operative site without replacement of the dorsum of the skull. Postoperative care included intramuscular penicillin for the first few days and intraperitoneal isotonic saline until the animals began drinking spontaneously. Pelleted and powdered purina checkers were supplied *ad lib.* In 2 groups, one week of postoperative recovery was allowed and in one group 5 weeks. One ml of propylthiouracil suspension in 10% acacia containing 30 mg/ml was then injected subcutaneously once daily at rotated sites. Control animals received only the propylthiouracil injections. All animals were killed 11 days after beginning propylthiouracil treatment and thyroids, adrenals, uteri and ovaries weighed on a 200 mg torsion balance. The skin and mandible were removed from the skulls of the decorticate animals. After a few days fixation in formalin, the brains were then examined to determine the extent of cortical ablation.

*Results.* The autopsy data are summarized in Table I. No great difference was found between the goitrogenic response of the control and operated animals nor did the other endocrine organs examined show striking deviation of the operated from control animals. Although gross inspection of the brains of the operated animals revealed that ablation of the neocortex was not always quite complete, no correlation existed between deficiency of cortical tissue and size of the thyroid or other organs examined. It is possible that the slightly smaller thyroid weights of the operated animals reflect their inferior nutritional status.

This study failed to demonstrate any significant loss of hypophyseal function after removal of the neocortex in the rat. Previous studies by Davis(5) indicated that removal of the neocortex in this species did not interfere with mating, pregnancy or parturition. Since lesions of the hypothalamus can lead to such profound alterations in pituitary func-

* Supported in part by U.S.P.H.S. Grant.

† Present address: Dept. of Medicine, Univ. of Oregon Medical School, Portland.

TABLE I. Body and Organ Weights of Decorticate Rats Treated with Propylthiouracil.*

| Group | No. animals | Initial body wt (g) | Final body wt (g) | Thyroid (mg) | Ovaries (mg) | Adrenals (mg) | Uterus (mg) |
|---|---|---|---|---|---|---|---|
| I | 6 | 198 ± 4.8† | 208 ± 5.2 | 15.1 ± .6 | 42.3 ± 5.8 | 63.2 ± 2.2 | 271.1 ± 27.8 |
| II | 5 | 207 ± 4.6 | 198 ± 7 | 40.5 ± 1.5 | 48.7 ± 6.2 | 60.6 ± 2.9 | 290.7 ± 32.8 |
| III | 4 | 196 ± 3.9 | 164 ±10 | 29.2 ± 1.0 | 35.8 ± 2.7 | 59.8 ± 3.3 | 269.3 ± 13.7 |
| IV | 4 | 201 ± 4.1 | 162 ± 9.4 | 32.2 ± 1.2 | 38.3 ± 2.9 | 58.5 ± 4.1 | 271.1 ± 18.2 |
| V | 3 | 199 ± 3.2 | 162 ± 4.0 | 31.3 ± 2.4 | 37.2 ± 3.2 | 53.3 ± 3.0 | 251.9 ± 15.1 |

* Group I = Untreated controls; II = propylthiouracil-treated controls; III & IV = decorticate rats allowed one wk for post-operative recovery; V = decorticate rats allowed 5 wk for post-operative recovery. Groups II through V inj. with propylthiouracil suspension for 10 days and killed on 11th day.

† All figures given as mean ± stand. error.

tion, it appears that, whatever extra-hypothalamic nervous pathways may be concerned in modifying hypothalamic activity, none of these are mediated primarily by the neocortex.

*Summary.* Removal of the neocortex in female rats did not greatly affect the goitrogenic response to propylthiouracil administration nor the weights of the adrenals, ovaries or uteri.

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Received December 3, 1956. P.S.E.B.M., 1957, v94.

## *In vivo* Synthesis of Ascorbic Acid by the Alloxan Diabetic Rat. (23014)

J. V. STRAUMFJORD, JR.* AND E. S. WEST

*Department of Biochemistry, University of Oregon Medical School.*

The capacity of the albino rat to synthesize ascorbic acid has been known for many years, but only recently has the mechanism of synthesis been partially clarified. King and associates(1) reported that certain compounds accelerate synthesis and excretion of ascorbic acid. One of these compounds, Chloretone, has been used extensively in the study of precursors and mechanism of ascorbic acid synthesis in the rat. Chloretone is capable of increasing urinary excretion from a basal rate of 0.3 mg to 50 mg/24 hours. The evidence that glucose is a precursor for ascorbic acid in the rat is conclusive. Jackel, Mosbach, Burns and King(2) injected uniformly labeled $C^{14}$ glucose into Chloretone treated rats and found 0.3% conversion into ascorbic acid. Degradation studies indicated that ascorbic acid was also labeled uniformly. This work suggested that glucose is the precursor and that the entire molecule is used. Horowitz, Doerschuk and King(3) injected D-glucose-1-$C^{14}$ into Chloretone stimulated rats and found approximately 56% of the total activity in carbon 6 of the excreted ascorbic acid, the position expected according to theory. Horowitz and King(4) similarly showed conversion of glucose-6-$C^{14}$ to ascorbic acid-1-$C^{14}$. More recently Burns and Mosbach(5) have shown that the normal rat as well as Chloretone-treated animal converts D-glucose-1-$C^{14}$ to ascorbic acid-6-$C^{14}$.

The purpose of this study was to ascertain whether or not the synthesis of ascorbic acid by Chloretone-stimulated rats is affected by the diabetic state in which the primary meta-

* Present address: Department of Surgery, State University of Iowa, Iowa City.

TABLE I. Urinary Excretion of Ascorbic Acid by Rats before and after Addition of Chloretone to the Diet.*

| | Fasting blood sugar, mg % | Urinary ascorbic acid (mg/24 hr) | |
|---|---|---|---|
| | | Milk | Milk + Chloretone |
| Control (11) | 81 ± 15 | .76 ± .47 | 22.03 ± 9.89 |
| Alloxan diabetic (12) | 284 ± 119.5 | 3.40 ± 1.21 | 25.3 ± 5.65 |
| " resistant (6) | 111 ± 24 | 2.03 ± .51 | 23.5 ± 5.04 |

Statistical Comparison of Ascorbic Acid Excretion.

| | P† | |
|---|---|---|
| | Before Chloretone | After Chloretone |
| Alloxan diabetic *vs* control | <.005 | >.10 |
| Alloxan resistant *vs* control | " | " |
| Alloxan diabetic *vs* alloxan resistant | <.025 | |

* Values given = mean ± stand. dev. † Level of significance.

bolic deficiency apparently is represented by decrease in the hexokinase reaction and the conversion of glucose to glucose-6-phosphate.

*Methods.* Twenty-seven male and 4 female rats, weighing approximately 190 g and of the Sprague-Dawley strain, were fasted for 24 hours and then maintained on a diet of evaporated milk until excretion level of ascorbic acid was stabilized and a suitable number of control levels obtained. Animals were kept in metabolic cages and urine collected over 24-hour periods. Three groups of animals were employed: 1. Normal; 2. Alloxan diabetic; 3. Rats given alloxan but which maintained blood sugar levels below 140 mg %. The rats in group 2 were made diabetic by intramuscular injection of 0.1 ml of 10% alloxan solution/100 g body weight. After excretion of ascorbic acid stabilized, 20 mg of Chloretone was added daily to the diet in all groups of animals. Excretion of ascorbic acid in urine was determined on the 1st, 2nd, 4th, and 6th days after addition of Chloretone. There was no appreciable increase in excretion after the 6th day. Ascorbic acid was determined by the Bolin and Book(6) modification of the Roe and Keuther(7) method. Urine was collected as described by Schwartz and Williams(8). Somogyi's (9) method was used for blood sugar determination.

*Results.* The results for 11 normal control rats are shown in Table I and demonstrate a mean excretion of ascorbic acid of 0.76 ± 0.47 mg/24 hrs. After 6 days of evaporated milk plus Chloretone, they excreted a mean of 22.03 ± 9.89 mg/24 hrs. The 12 alloxan diabetic rats excreted a mean of 3.4 ± 1.2 mg/24 hrs while on evaporated milk and after 6 days of Chloretone, excreted a mean of 25.3 ± 5.65 mg/24 hrs. The third group, consisting of 6 rats, had been injected with alloxan but did not show much elevation in their fasting blood sugar. This group was arbitrarily called alloxan-resistant and was included with the series because they demonstrated an increase in the ascorbic acid excretion while on evaporated milk, giving a mean value of 2.03 ± 0.51 mg/24 hrs. The mean excretion after 6 days of evaporated milk plus Chloretone was 23.5 ± 5.04 mg/24 hrs in this group. Mean fasting blood sugar for the normal control rats was 81 ± 15 mg/100 ml, for the alloxan diabetic animals 284 ± 119.5 mg/100 ml, and for the alloxan-resistant rats 111 ± 24 mg/100 ml.

Statistical comparison of the 3 groups is shown in the table. It is apparent from the level of significance that the alloxan diabetic and normal rats were in statistically different groups relative to excretion of ascorbic acid while on evaporated milk. This was also true of the alloxan-resistant and normal rats. The level of significance is somewhat lower when the alloxan diabetic and alloxan-resistant groups are compared, but the difference is statistically significant. There was no statistical significance between all 3 groups after adding Chloretone to the diet.

*Discussion.* It is evident that the alloxan

diabetic rat is capable of synthesizing as much ascorbic acid as the normal rat under the stimulus of Chloretone. This suggests that the usual pathways of glucose utilization which are blocked or inhibited in the alloxan diabetic rat are not necessary for the synthesis of ascorbic acid. Thus, the conversion of glucose to glucose-6-phosphate apparently is not an essential reaction for the synthesis of ascorbic acid.

The excretion of ascorbic acid in the 3 groups is much different while on evaporated milk only. The alloxan diabetic rat excreted far greater quantities of ascorbic acid than did the normal rat. Among the possible explanations for this observation are the following: (1) Alloxan has a toxic action on the tubular reabsorption mechanism in the kidney resulting in increased ascorbic acid excretion and stimulus to synthesis. (2) There is competition in the tubular reabsorption mechanism between glucose and ascorbic acid with preference to reabsorption of glucose, thus effecting increased ascorbic acid excretion and, in turn, stimulating increased basal synthesis. Competition between glucose and ascorbic acid reabsorption has been demonstrated in the dog by Selkurt(10). (3) Increased blood sugar by mass action effect stimulates increased synthesis. There is some evidence to show that added glucose feeding does not increase synthesis of ascorbic acid. Perhaps the sustained high level of blood sugar in the diabetic does increase the rate of synthesis. Any one of these explanations would also explain the observation that the alloxan-resistant animal excreted more ascorbic acid than did the normal, but less than the diabetic rat while on evaporated milk only. It seems that the second hypothesis may represent the most likely explanation.

*Summary.* Synthesis of ascorbic acid is not impaired in the alloxan diabetic rat, indicating that the hexokinase reaction is not necessary for this synthesis. Ascorbic acid excretion by the alloxan diabetic rat and the alloxan-treated but nondiabetic rat is greater than for the normal rat while on evaporated milk only.

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Received December 10, 1956. P.S.E.B.M., 1957, v94.

# Functional Survival of Ovarian Homografts within Millipore Filter Chambers in the Castrate Rat.* (23015)

Somers H. Sturgis and Hector Castellanos. (Introduced by R. O. Greep)
*Peter Bent Brigham Hospital, Boston, Mass.*

Homografts in rats are known to survive and function unless the animals have been immunized by previous transplants. After such immunization, grafts become rapidly replaced by fibroblasts and soon nothing but a scar is left. Algire and associates(1) however, found that when they excluded the host's cells from contact with their transplants by embedding the latter between two special filters, they obtained satisfactory survival. For this purpose they used millipore filters† with pore sizes of 0.45 and 0.8 $\mu$. Mammary carcinoma transplants with a survival of only 5.8 days in immunized hosts were shown to be viable at least 60 days after transplantation in such a system.

This suggested to us the possibility that homografts of endocrine glands in primates might perhaps be protected from destruction if similar filters were used to prevent leucocytic infiltration and vascularization. To maintain function as well, the filters would have to allow the inward passage of tropic hormones from the pituitary and the outward elimination of secretions from the grafted transplants. This is a preliminary report with ovarian homografts in the non-immunized rat.

*Material and methods.* Millipore filters are made of intermeshed cellulose ester filaments having extremely uniform pore size that are manufactured in various dimensions. Chambers for the grafts were constructed similar to those used by Algire, by cutting thin cross-sections from a ½ inch lucite tube. A section of the filter membrane was cut and glued with Duco cement to one side of a cross-section, and then after sterilization in ethylene oxide gas, 2 such sections were glued together after inclusion of a desired piece of fresh tissue under aseptic conditions. Preliminary tests showed that pituitary gonadotropins contained in urine, and plasma‡ of a young human castrate could be drawn by suction through the HA millipore filter (pore

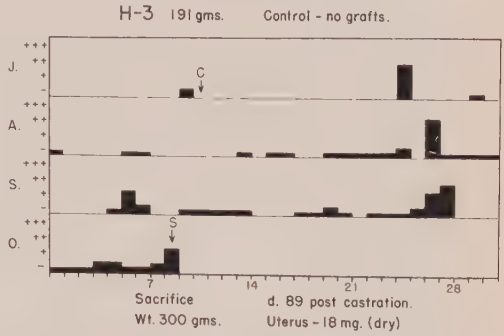


FIG. 1. Cornification of vaginal smear of castrate control. In 89 days after castration, only occasional and sporadic positive smears (++ or +++) were found, probably due to traumatization. Uterine weight at autopsy 18 mg. C = castration; G = graft; S = sacrifice; J, A, S, O = July, Aug., Sept., Oct.; baseline in days of month.

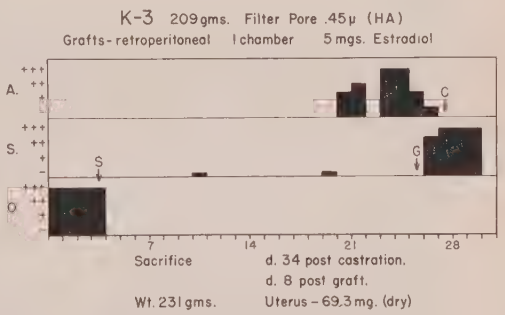


FIG. 2. Vaginal cornification is maintained at full estrus when estradiol crystals are embedded in an HA millipore filter chamber one month after castration. At sacrifice 8 days later the uterus weighed 69.3 mg.

* This work was supported in part by Am. Cancer Soc., Mass. Div. Inc., also by Am. Cancer Soc. Institutional Grant to Harvard University and N.I.H. Grant.

† Millipore filter membranes were obtained from the Millipore Filter Corp., Watertown, Mass.

‡ Estrogen-free human plasma was contributed through the courtesy of Dr. H. Antoniades of Protein Foundation, Jamaica Plain, Mass. It was assayed after filtration by its effect on uterine weight of immature mice according to standard procedures.

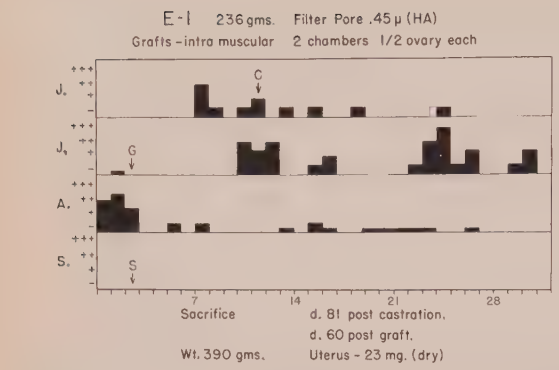


FIG. 3. Cornification is produced only temporarily by ½ an ovary embedded in an HA filter chamber after castration. G = date of grafting.

size 0.45 μ) so we chose this membrane for the experiments below. Adult female albino rats weighing approximately 200 g were observed through one or 2 estrous cycles by daily vaginal smears. Castration was performed and one month later, sections of ovary from another adult rat were introduced within millipore filter chambers in various positions — intramuscular, intraperitoneal and retroperitoneal — and in varying sizes. Vaginal smears were again followed up to about 60 days when the animals were sacrificed, the uteri dissected and their dry weight recorded. In each group of experiments control animals were prepared with no tissue transplants for comparison. Histologic sections stained with H and E were then prepared from the transplants recovered at sacrifice.

*Results.* Control animals invariably showed the expected atrophic vaginal smear of anestrus. After 60 days, the dry uterine weight ranged from 16-20 mg (Fig. 1). When 5 mg estradiol crystals were enclosed in an HA filter chamber in a castrate, the vaginal smear showed a full estrous effect from the day after operation through the next 8 days, and the uterine weight was maintained at over 60 mg (Fig 2). Two chambers each containing ½ an ovary produced a vaginal smear response suggesting some functional survival for a few weeks, but this dwindled away, and at sacrifice the uterus was again atrophic (Fig. 3).

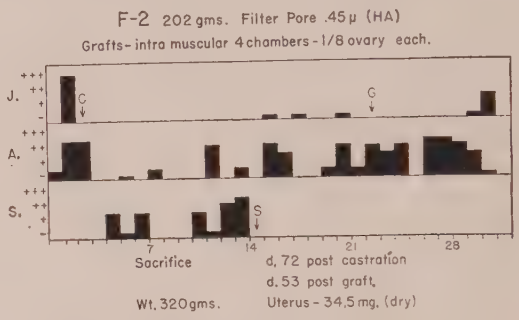


FIG. 4. Homografts of approximately ⅛ of ovary showed some evidence of steroid production in vaginal smears for 53 days when transplanted intramuscularly, and uterine weight at sacrifice was 34 mg.

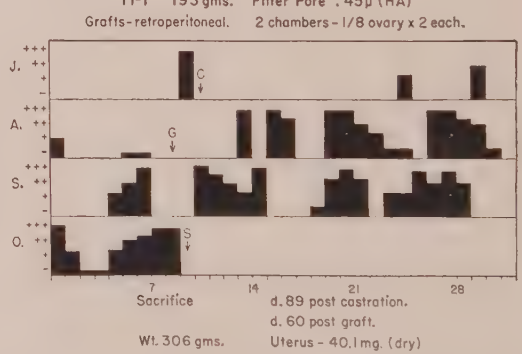


FIG. 5. Transplantation of ⅛ ovary in filter chamber retroperitoneally one month after castration produces cycles of cornification approximating at interval of 4½ days typical for estrous cycle in this animal. Uterus weighed 40.1 mg at sacrifice.

TABLE I. Uterine Dry Weights in Castrate Rats with Filter Chamber Ovarian Grafts.

| Preparation | Site | Rat wt | Days P castrate | Days P graft | Filter, pore μ | Uterus wt, mg |
|---|---|---|---|---|---|---|
| Intact | | 189* | | | | 63.5* |
| H3—control | | 300 | 89 | | | 18.0 |
| Estradiol | RP | 231 | 34 | 8 | .45 | 69.3 |
| H2—4 × ⅛ ov. | " | 274 | 89 | 60 | .8 | 16.0 |
| E1—2 × ½ ov. | IM | 390 | 81 | 60 | .45 | 23.0 |
| F2—4 × ⅛ ov. | " | 320 | 72 | 53 | .45 | 34.5 |
| H1—4 × ⅛ ov. | RP | 306 | 89 | 60 | .45 | 40.1 |

* Avg.

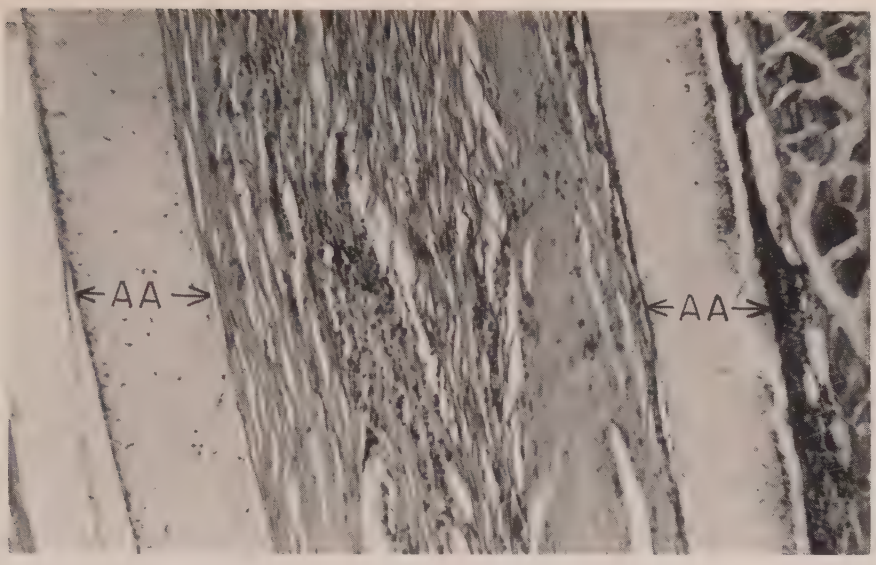


FIG. 6. Section through an AA filter chamber (pore size 0.8 $\mu$) to show membranes on each side infiltrated with cellular debris.

When 1/8 of an ovary was used, the vaginal smear and uterine weight were more satisfactory (Fig. 4). In both the latter experiments the chambers were embedded in the muscles of the back. The best results were obtained with chambers containing 1/8 fragments of ovary placed retroperitoneal. After 60 days a continuing estrous cycle approximating the 4½ days typical for this strain was seen, and the uterine weight showed definite estrogenic support (Fig. 5).

Table I summarizes the dry uterine weights of intact and castrate control rats and the experimental animals recorded in Fig. 1-5.

Histologic sections demonstrate that the AA filter (pore size 0.8 $\mu$) becomes infiltrated with cellular debris (Fig. 6) in contrast to the HA filter (pore size 0.45 $\mu$) which remains clear of cell fragments (Fig. 7). Although ovarian function appeared to be maintained

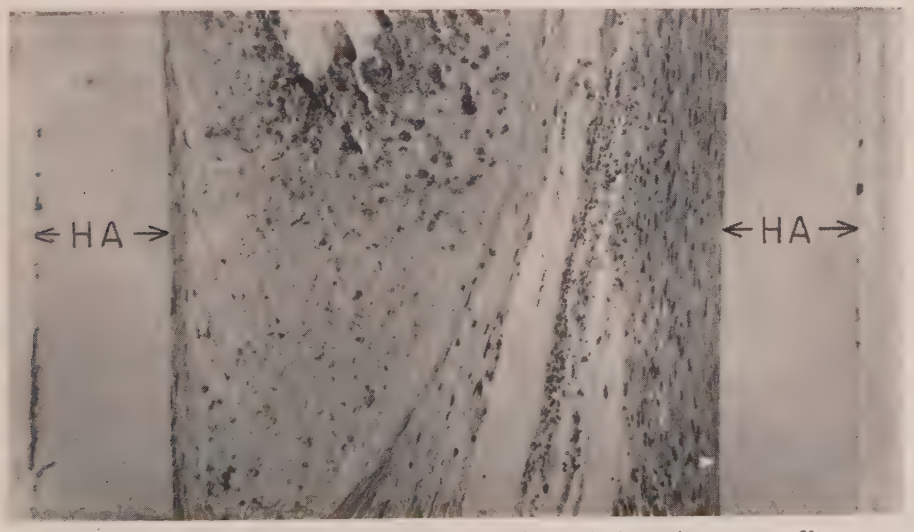


FIG. 7. Section through an HA filter chamber (pore size 0.45 $\mu$) to demonstrate filters on each side entirely clear of cell fragments. Ovarian graft within is non-vascularized; there is no leucocytic infiltration.

in chambers of the latter (Fig. 5) yet the sections in Fig. 7 show a complete degeneration of follicles and ova 60 days after grafting.

*Conclusions.* Homografts of the rat ovary do not depend on vascularization to maintain the production of estrogenic substances up to 60 days. Semipermeable millipore filter membranes were used to form a chamber in which to embed ovarian transplants in previously castrated recipients. These chambers excluded leucocytes and prohibit vascularization, yet permitted passage of gonadotropic hormones. Vaginal smears and uterine weights demonstrated the production of estrogens from these grafts two months later. The functional survival of similar preparations in the monkey is now under investigation.

The authors are grateful for technical assistance of Dix Campbell in preparing the filter chambers.

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Received December 14, 1956. P.S.E.B.M., 1957, v94.

## Hypophysectomy and the Tail Darkening Reaction in *Xenopus.** (23016)

JOSEPH T. BAGNARA.† (Introduced by Arthur R. Kemmerer)
*Department of Zoology, State University of Iowa.*

It has long been known that if the hypophyseal placode is removed from amphibian embryos, the resulting larvae will be very pale in appearance and vary in color in different species from white or silver, to golden yellow(1,2). Pallor generally results from persistent contraction of epidermal melanophores and expansion of both dermal and epidermal guanophores(2). It is therefore surprising to observe that in hypophysioprivic *Xenopus* larvae, melanophores of the tail are capable of considerable expansion or contraction under appropriate conditions. The experiments reported here were designed to study the influence of the hypophysis on the behavior of some of the chromatophores of *Xenopus* larvae, and to gain some insight into the peculiar activity of melanophores in the tail.

*Methods.* All tadpoles were raised from naturally spawned eggs of *Xenopus laevis* Daudin. At an early tailbud stage, the hypophyseal placode was removed from 378 embryos. Judging from the number of pale larvae resulting, removal of the hypophysis was accomplished in about 85% of the operated animals. Only about 25% of these larvae survived after 2 weeks, because of severe damage to their mouth parts.

Melanophore counts were made in the ventral fin of both normal and hypophysioprivic larvae of the same egg batch. An area about one-third the distance from the anterior margin of the pigmented region of the tail and midway between fin and somites was selected for the counts. These were made using an ocular micrometer on 6 larvae from each group. Under magnification of 45X, melanophores enclosed in a square, one-sixteenth of a square cm in size, were counted for 25 squares and the average number per square was then determined for each tadpole.

*Results.* In hypophysioprivic larvae a very great reduction in number of melanophores was noted in all regions except the tail, and those that were present were maximally contracted. This was especially true of melanophores present on thymus, nerves, and blood vessels (Fig. 1, 2). In the tail, melanophore number was not greatly influenced by hypophysectomy (Fig. 3, 4, 5). A reduction of about 30% occurred in the number of tail melanophores in hypophysioprivic tadpoles. This is a remarkably small percentage compared with the great reduction observed in other parts of the tadpole.

In normal *Xenopus* larvae, guanophores are

* Supported by grant from Nat. Science Fn.

† Present address: Dept. of Zoology, Univ. of Arizona.

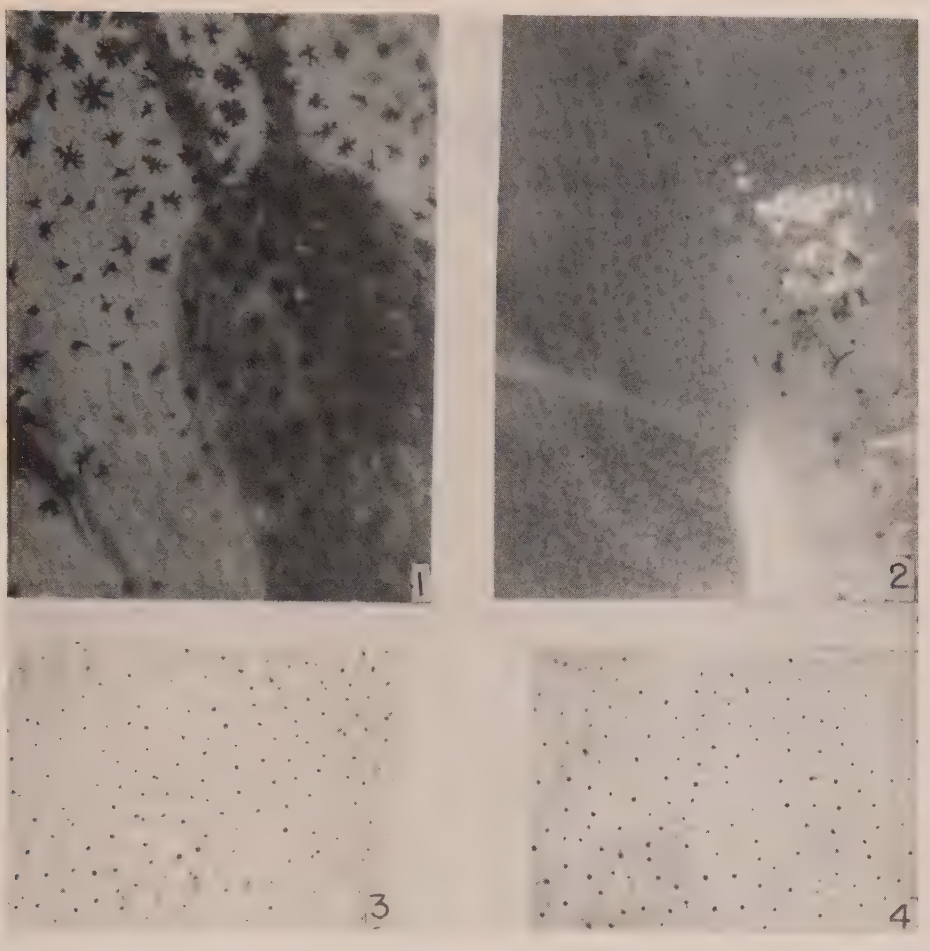


FIG. 1. Anterior head region of normal *Xenopus* larva showing distribution of melanophores over brain, in skin, and on deep nerves and blood vessels. X45.

FIG. 2. Anterior head region of a hypophysioprivic *Xenopus* larva showing reduction in number of melanophores over brain, in skin, and on deep nerves and blood vessels, and the presence of guanophores. X45.

FIG. 3. Contracted melanophores in ventral fin of normal *Xenopus* larva. X60.

FIG. 4. Contracted melanophores in ventral fin of hypophysioprivic *Xenopus* larva showing only slight reduction in number of melanophores. X60.

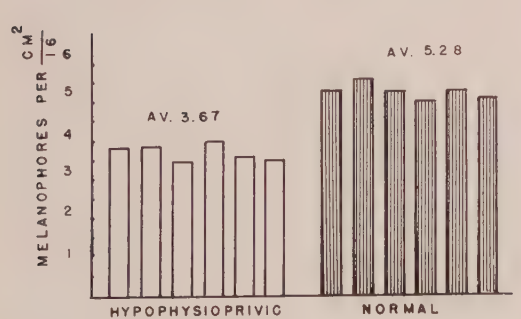


FIG. 5. Diagram showing relative number of melanophores in ventral fin of 6 hypophysioprivic and 6 normal *Xenopus* larvae.

restricted to eyes and abdomen(3). In hypophysioprivic larvae, however, they were present as large glistening bodies in the integument of head and tail (Fig. 2, 6, 7).

When either normal or hypophysioprivic tadpoles were placed in the dark at room temperature for about 30 minutes, tail melanophores expanded greatly, causing the tail to appear very black. When tadpoles were returned to the light, these melanophores contracted and within 5 or 6 minutes, the tails regained their former light appearance. Be-


FIG. 6. Hypophysioprivic *Xenopus* larva showing typical paleness and presence of guanophores in the tail. X1.5.
FIG. 7. Normal *Xenopus* larva. Note darkness, and lack of guanophores in tail. X1.5.

cause tail darkening reaction obviously involves reception of light, the eyes were removed from 5 hypophysioprivic and 10 normal larvae. During the next few days, these larvae were tested repeatedly and in all cases displayed the same positive tail darkening reaction. To determine whether direct nervous mediation of tail melanophores might be a factor in the tail darkening reaction, the spinal cord and lateral line nerves of the tail were severed in 4 normal larvae. A disappearance of the sinusoidal wave pattern of tail movement occurred, but ability of tadpoles to carry out the tail darkening reaction was in no way impaired by the operation.

With the eyes and direct ennervation eliminated as important elements in the tail darkening reaction, it seemed possible that tail melanophores might be directly stimulated by light. To explore this possibility, tails were cut from at least 50 normal larvae and placed in Petri dishes containing tap water. Some dishes were placed in the dark, while others serving as controls were left illuminated. After 30 minutes, the tails were brought back into the light and were very dark due to expansion of their melanophores. Just as in intact tadpoles, 5 or 6 minutes after exposure to light, the tails became pale and appeared no different from the controls. This reaction could be consecutively repeated at least 3 times.

In a similar experiment, a microscope ocular was replaced with a small light source so that a narrow beam of light could be passed through the microscope and focused on the stage. By using this microscope in a dark room, it was possible to illuminate only a small region of tail mounted on the stage. In such experiments, melanophores in the illuminated region remained contracted, while those in the dark area displayed the typical tail darkening reaction (Fig. 8).

*Discussion.* Just as in most amphibians, removal of the hypophysis of *Xenopus* results in marked reduction in number of melanophores over the entire tadpole. The fact that this reduction is relatively small (30%) in the tail, is probably related to the tail darkening effect, which seems to operate independently of the hypophysis. This does not imply that tail melanophores are not stimulated by the hypophysis, in fact, Thing(4) uses expansion of tail melanophores in *Xenopus* as an index in the assay of the chromatophorotrophic hormone.

Appearance of guanophores in such regions as the tail and head of hypophysioprivic *Xen-*

FIG. 8. Tails of normal *Xenopus* larva; upper, under lighted conditions; middle, tail darkening reaction except for center part which was left illuminated; bottom, at height of tail darkening reaction. X3.

*opus* larvae possibly reflects a general characteristic of anurans wherein the chromatophorotrophic hormone of the hypophysis elicits a guanophore inhibition. Hence, guanophores are contracted in normal larvae of *Rana,* but are expanded in hypophysioprivic tadpoles. This inhibition is perhaps of even greater potency in *Xenopus* and could account for the lack of differentiation of guanoblasts in the trunk and tail, as reported by Stevens(3).

The apparent direct stimulation of tail melanophores by light suggests that these cells possess a photochemical system. Additional evidence for this hypothesis can be derived from the fact that contraction of melanophores in the dark reacted tail occurs within 6 minutes after removal from the dark. The 30 minute period required for tail darkening could correspond with the synthesis of a photochemical substance, while the 5 or 6 minute periods might relate to its rapid inactivation or destruction by light. Support for this hypothesis is gained from some experiments now in progress which indicate that these melanophores can dark adapt.

Hypophysectomy has a profound effect on distribution of chromatophores in *Xenopus* larvae. A great reduction in number of melanophores occurs over the entire tadpole except in the tail where guanophores now appear. The tail melanophores can behave independently of the hypophysis and respond directly to stimulation by light.

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Received December 26, 1956. P.S.E.B.M., 1957, v94.

# Comparison of Methods for Quantitating Melanophore Responses.* (23017)

S. Deutsch, E. T. Angelakos, and E. R. Loew
*Department of Physiology, Boston University School of Medicine, Boston, Mass.*

Development of methods for quantitation of melanophore responses followed a proposal by Sulman(1) that the corticotropin molecule has inherent melanophore stimulating activity. The relationship between the melanophore stimulating (MS) and adrenal ascorbic acid depleting activities of corticotropin is still controversial. Recent evidence of a similarity in sequence of amino acids in both intermedin and corticotropin may possibly explain the MS activity of corticotropin preparations(2,3). The report of Lerner and coworkers(4) indicating an ability of intermedin preparations to stimulate melanin pigmentation in man has also increased the need for methods of assaying intermedin activity in physiological media. Recent reports(5,6) indicate that there is an increased melanophore stimulating activity of the blood in certain clinical conditions such as Addison's disease, Cushing's syndrome, rheumatoid arthritis, postoperative stress, pregnancy, and others. Because of the variability of melanophore responses(7), it is felt that only objective quantitative methods which yield data that can be treated statistically are acceptable for purposes of bioassay. Qualitative methods utilizing arbitrary scales having no mathematical basis, thus precluding statistical analysis, have been criticized as methods of bioassay(8). Methods for quantitating melanophore responses may be divided into 2 broad groups: those utilizing intact animals, and those which make use of isolated strips of adult frog or tadpole skin. Photoelectric measurement of the changes in light reflected from the surface of intact frogs or transmitted through isolated skin has been employed in quantitating the responses of frog melanophores. Schizume and coworkers(9) have measured the light reflected from isolated strips of frog skin in quantitating melanophore responses.

* Supported by grant from Parke, Davis and Co., Detroit, Mich.

The following study was carried out in order to compare an *in vivo* method previously reported(7) with an *in vitro* method first reported by Wright(10) and developed by Frieden and coworkers(11). Because the methods of preparation of pituitary extracts differ widely in various laboratories it was felt that only a comparison of the two methods using the same hormone preparation would be a true test of the precision, sensitivity, and reliability of the two methods.

*Materials and methods.* A detailed description of the *in vivo* method has been presented(7). For the *in vitro* method, frogs (*Rana pipiens*) were pithed, and the skin from both legs placed in Ringer's solution pH 7.13 at 23°C. Strips of skin were mounted between 2 pieces of plastic having holes 18 mm in diameter, through which light transmission could be measured. It was found that the Ringer's solution had to be changed twice at 20 minute intervals following removal of the skins from the animals. If this was not done the pigment within the melanophores dispersed after a short period of concentration. A transmission of 25 on the densitometer was chosen as the minimum transmission that would be allowed as a starting point. The melanophores were considered maximally contracted if, after preliminary washings, 2 transmission measurements taken 15 minutes apart did not differ by more than 2 units. Following the control measurements, a range of concentrations of Armour ACTH lot no. L60311 was added, and transmission measurements were taken 10, 20, and 40 minutes later. The skins were then washed twice at 15 minute intervals with Ringer's solution, and immersed in a "standard" concentration of ACTH. Transmission measurements were made 40 minutes after exposure to the "standard" solution. Groups of frog skins were immersed in a range of concentrations of 0.001 $\mu$g/ml to 0.05 $\mu$g/ml in a volume of 30 ml. The work was carried out in 2 series.

TABLE I. Effect of Corticotrophin (ACTH) on Light Transmission in Isolated Frog Skin.

| ACTH ($\mu$g/ml) | Light transmission readings: Initial | 40 min. | "Standard" .025 $\mu$g/ml | $\Delta_1/\Delta_2$* |
|---|---|---|---|---|
| | | Series 1 | | |
| .001 | † 32.3 | 28.1 | 24.4 | .53 |
| | ‡ 4.6 | 5.3 | 3.9 | .23 |
| .005 | 32.4 | 29.3 | 24.2 | .34 |
| | 6.9 | 5.6 | 5.6 | .52 |
| .025 | 34.4 | 30.8 | 28.5 | .59 |
| | 7.7 | 5.3 | 6.1 | .30 |
| .125 | 36.5 | 22.2 | 25.0 | 1.71 |
| | 8.4 | 10.0 | 7.1 | 1.01 |
| Control | 32.7 | 31.3 | | |
| | 8.9 | 5.8 | | |
| | | Series 2 | | |
| | | | .250 $\mu$g/ml | |
| .125 | 40.5 | 27.1 | 24.3 | .81 |
| | 4.1 | 8.7 | 9.2 | .16 |
| .250 | 38.6 | 21.0 | 18.1 | .73 |
| | 2.4 | 4.9 | 7.0 | .34 |
| .500 | 40.3 | 21.0 | 20.3 | .97 |
| | 3.3 | 8.9 | 10.2 | .17 |
| Controls | 43.0 | 41.0 | | |
| | 7.8 | 7.4 | | |

* $\Delta_1$ = Starting transmission, 40 min. response to dose. $\Delta_2$ = Starting transmission, 40 min. response to "standard" dose.
† Mean. ‡ Stand. dev.

One series of skins was subjected to concentrations of 0.001, 0.005, 0.025, and 0.125 $\mu$g/ml. A concentration of 0.025 $\mu$g/ml was used as the "standard" for this series. The second series of skins was treated with doses of 0.125, 0.250, and 0.50 $\mu$g/ml. The "standard" concentration for this series was 0.250 $\mu$g/ml. Groups of skins were placed in 30 ml of Ringer's solution and served as controls.

*Results.* Table I contains a summary of the data obtained. Mean starting values, mean transmission 40 minutes after exposure of the first concentration of ACTH, and the mean transmission 40 minutes after the second "standard" concentration of ACTH are presented. The difference in the light transmission before and after exposure to the first concentration ($\Delta_1$) and between the starting transmission and the response to the second concentration ($\Delta_2$), can be expressed as a ratio, ($\Delta_1/\Delta_2$), following the method of Frieden *et al.*(11). Table I shows the mean $\Delta_1/\Delta_2$ ratios. The results with $\Delta_1/\Delta_2$ ratios when the first concentration is the same as the "standard" concentration do not agree with those of Frieden *et al.*(11), who reported a mean ratio 0.95 $\pm$ 0.04 under such conditions. Generally it was found that using the same concentration, the response to the second exposure was higher than that obtained on first exposure even after repeated washings between exposures. In the first series the second response to 0.025 $\mu$g/ml was 1.7 times greater than the first response. Similarly a ratio of 1.3 between second and first exposures was obtained in the second series with 0.25 $\mu$g/ml. Since the variability was increased by expressing the response as the ratio, it was decided to use the 40 minute transmission measurements as the responses.

A log dose-response line was drawn between the concentrations of 0.025 to 0.250 $\mu$g/ml. Application of Bartlett's test indicated a homogeneity of the variances of these groups. It was concluded from this that the variance was independent of the response. Analysis of the variance was performed. Equation of the line was found to be $Y_x = 13.0\text{-}11.1 \log X$. The precision index lambda was calculated to be 0.60.

*Discussion.* A statistical comparison of the *in vitro* and *in vivo* methods is presented in Table II. With respect to the sensitivities of the two methods, the one using intact animals is twice as sensitive as that using isolated frog skin. When one considers the total amount of hormone needed for the *in vitro* method (30 ml) the *in vivo* method is 60 times more sensitive. The former method is less specific (12) and more susceptible to changes in pH, temperature, and ionic concentration(13). Similar considerations regarding sensitivity

TABLE II. Statistical Comparison of *In Vivo* and *In Vitro* Methods of Quantitating Melanophore Responses.

| | *In vivo* | *In vitro* |
|---|---|---|
| Sensitivity ($\mu$g) | .064 | .125 |
| Range of response ($\mu$g) | .064–6.4 | .125–.250 |
| Mean coef. of variation | 10.5 | 35.7 |
| Regression line | | |
| Slope | 4.0 | 11.1 |
| Variance of error | 2.82 | 44.6 |
| Lambda | .39 | .60 |

and specificity would apply to the *in vitro* method reported by Schizume and associates (9).

The dose range for submaximal responses was 100 fold in the method using intact frogs. With the isolated preparations the range was only 2 fold. A long range for submaximal responses is considered desirable for a bioassay since it permits comparison between standard and unknown even though their potencies may vary considerably.

As a measure of the variability of the response to a given dose, the mean coefficient of variation was 3.4 times greater in the method using isolated frog skin.

With respect to the regression line, the slope obtained with the *in vitro* method was greater than that with the *in vivo* method. However the variance of error of the latter method was considerably less than that of the former. Thus, lambda, the precision index, was greater for the *in vitro* method, and approximately 2.4 times as many determinations would have to be carried out using this method in order to obtain an error of 10% or less.

*Conclusions.* It is concluded that the method employing intact frogs is superior to that in which strips of frog skin are used for assay of melanophore stimulating hormone.

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Received January 22, 1957. P.S.E.B.M., 1957, v94.

## Biliary Excretion of Bile Acids and Cholesterol in Bile Fistula Rats. Bile Acids and Steroids.*† (23018)

STEN ERIKSSON (Introduced by O. Wintersteiner)
*Department of Physiological Chemistry, University of Lund, Sweden.*

It has been demonstrated that bile acids are the main metabolic end products of cholesterol in the rat (Byers and Biggs(1), Bergström(2), Chaikoff and Dauben(3)). Rat bile contains taurocholic and taurochenodesoxycholic acid (Bergström and Sjövall(4)). Several authors have studied excretion of bile acids and cholesterol in the bile duct cannulated rat. Friedman, Byers and Michaelis (5) found that excretion of cholic acid and cholesterol in bile gradually decreased during the first 2 days following cannulation. Thompson and Vars(6a,b) however, studying the influence of altered thyroid activity upon excretion of cholic acid found that the biliary level of this substance rose following cannulation and reached a fairly constant plateau on third or fourth postoperative day. Recently Wysock, Portman and Mann(7) reported studies on nutritional effects upon biliary excretion of cholic acid and dihydroxycholanic acids. Their results, however, were based entirely upon determinations of

* Preliminary report was read at meeting of the Swedish Biochem. Soc., Uppsala, Jan. 27, 1956(10).

† This work has been supported by grant from Medical Faculty, University of Lund.

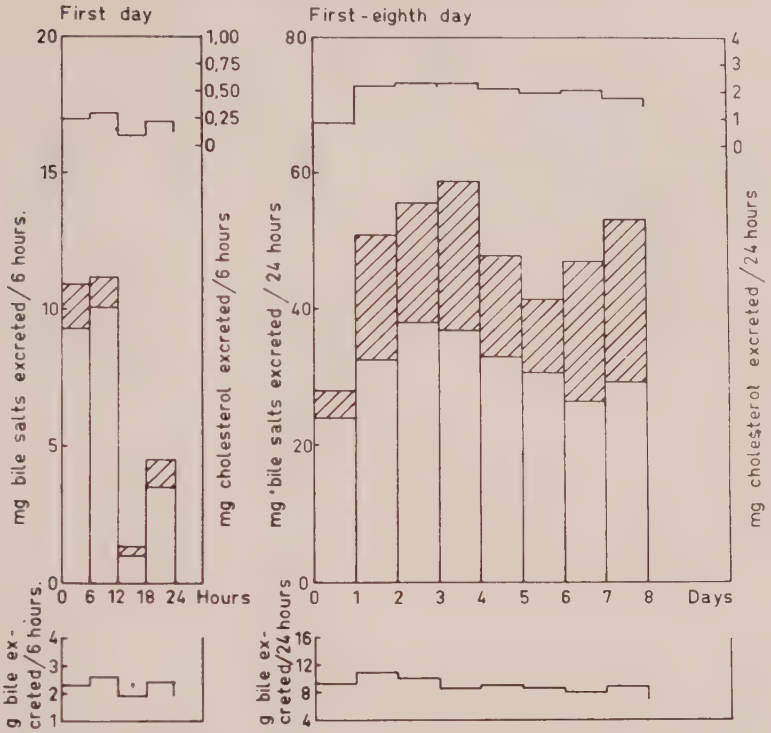


FIG. 1. Bile fistula rat 15 (230 g). Excretion of sodium taurocholate □, sodium taurochenodesoxycholate ▨ and cholesterol during 8 days. Values are expressed as mg/6 hr during the first day and as mg/24 hr during the following days.

bile acids in bile collected during the first 24 hours after operation.

The aim of the present investigation was to undertake a detailed study of excretion of taurocholic and taurochenodesoxycholic acid and cholesterol in the bile duct-cannulated rat. The methods developed by Sjövall(8) and Sjövall and Eriksson(9) make a direct, specific and quantitative microdetermination of both these acids possible.

*Methods.* White female or male rats of the institute stock, (weight: 200-300 g), were fed a diet consisting mainly of oats and white bread prior to operation. The common bile duct was cannulated and the animals were then kept in restraining cages during the experiment. Following cannulation, the animals were given white bread and 0.9% NaCl *ad libitum*. Bile was collected for various time intervals, usually for 6 hours, during the first day and later in 24 hour portions. Total time of collection ranged from one to 2 weeks. After collection, the bile was weighed and made up to definite volume with ethanol. Taurocholic and taurochenodesoxycholic acids were determined as their sodium salts by the methods developed by Sjövall(8). For paper chromatographic separation of the 2 acids, the ascending method with 70% formic acid as stationary and isoamylacetate heptane 85:15 as moving phase was used. Equilibration prior to running (15-20 hours), the chromatograms were found unnecessary. Seventy % formic acid gave more distinct spots than the 70% acetic acid previously used.‡ *Cholesterol in bile* was determined by a modification of the method of Abell *et al.*(11) for determination of total cholesterol in serum. One ml samples of the 24 hour bile diluted to 50 ml with ethanol, were measured into 12 ml glass stoppered centrifuge tubes. One ml ethanol and 1 ml 33% KOH was added to each tube. The stoppered tubes were kept in water bath at 60°C for 45 min-

‡ To be published.

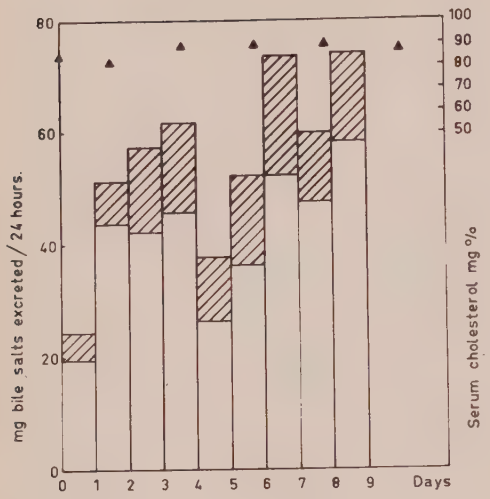


FIG. 2. Bile fistula rat 20 (290 g). Total serum cholesterol determined prior to operation and at various intervals after operation. Sodium taurocholate □ sodium taurochenodesoxycholate ▨.

utes. After cooling to room temperature 2 ml of water and 5 ml of petroleum ether were added and the tubes shaken vigorously for 1 minute. After 2 clear layers had formed 3 ml of petroleum ether was pipetted off and another 3 ml of petroleum ether added and the extraction procedure repeated in the same manner. The petroleum ether was evaporated to dryness in nitrogen atmosphere. Standards were run through the procedure along with the samples. The colour reaction used was a modified Tscgufaeff reaction as described by Dam *et al.*(12). The procedure described gave a 95-105% recovery of added cholesterol in the region 12-60 μg added to sample containing about 60 μg. *Total serum cholesterol* was determined according to Abell *et al.*(11) using the colour reaction described above. Blood samples (100-150 μl) were obtained from a cut on the tail. The determination was performed using 50 μl of serum.

*Results.* In Fig. 1 a typical excretion pattern is shown. During the first 18-24 hours after operation there is always a fall in excretion of taurocholic and taurochenodesoxycholic acid. A minimum generally occurs between 12 and 18 hours after operation. Following this minimum there is a rapid increase in amount excreted, and a fairly constant level is reached by the second or third day. During subsequent days total excretion of bile acids ranges between 40 and 60 mg/day. The major part, approximately 75%, is taurocholic acid. Excretion of cholesterol during the first day was about 1 mg and increased during the following days to about 2 mg/day. Amount of bile excreted usually decreases somewhat after the first day of collection (10-15 g/day). Fig. 2 shows the excretion pattern of a second rat. This Figure also shows that serum cholesterol remains practically unchanged during the time of experiment (9 days).

Thompson and Vars(6) in their work on biliary excretion of cholic acid in rats with modified thyroid activity noted low and erratic levels of cholic acid during the first day following cannulation. Cholic acid level then reached a fairly constant plateau. Their values on cholic acid are in agreement with ours. The low level of cholic acid was ascribed to hepatic injury at operation and plateau values were thought to represent normal formation of cholic acid in the intact animal. Friedman and Byers(5) noted decreasing values during the first 2 days after operation but did not follow excretion beyond 2 days. In our experiments we found a constant excretion pattern consisting of essentially 2 parts; during the first 12 or 24 hours following cannulation we observed a fall in excretion of both taurocholic and taurochenodesoxycholic acid. The amount of bile acids excreted in this time interval obviously represents excretion of preformed bile acids present in the enterohepatic circulation. From Fig. 1-3 it is possible to obtain an approximate value of the taurocholic acid pool. The amount of sodium taurocholate excreted during the first 18 hours varies between 6.1 and 8.8 with a mean value of 7.2 mg/100 g bodyweight. In another investigation under way, we determined the size of the taurocholic acid pool in the rat by using isotope dilution *in vitro* and *in vivo*. Preliminary data indicate the size of the pool to be about 12 mg sodium taurocholate in a rat weighing 200 g.§

Lindstedt and Norman(13) have estimated

§ To be published.

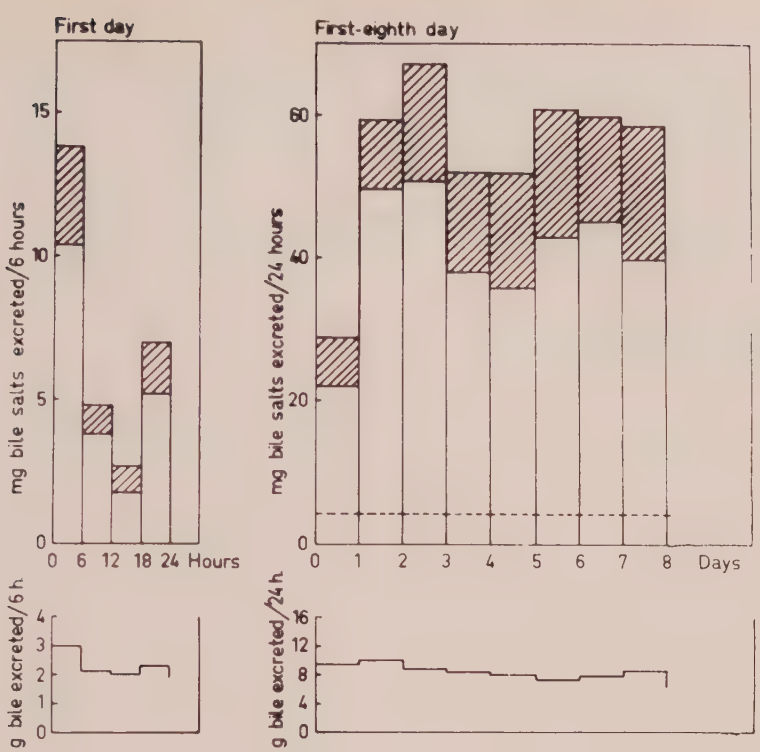


FIG. 3. Bile fistula rat 14 (260 g). Excretion of sodium taurocholate □ and sodium taurochenodesoxycholate ▨. Values are expressed as mg/6 hr during the first day and as mg/24 hr during following days. Broken line shows magnitude of taurocholic acid synthesis in the normal rat without a fistula.

the half lives of bile acids in the intact rat to be about 2.5 days. Using the preliminary figure of bile acid pool of 12 mg in a 200 g rat, the daily synthesis of taurocholic acid would be 3.2 mg. This means a synthesis of 0.12 mg/hour. Using this figure one obtains a value of 2.2 mg for synthesis during the first 18 hours assuming unchanged rate of synthesis. Subtracting this value from 14.4 mg one obtains 12.2 mg sodium taurocholate, which would thus represent the amount present in the enterohepatic circulation. The amount in a 200 g rat would consequently be 12.2 mg, a figure in good agreement with the value for the pool cited above. The second part of the excretion curve (comprising excretion from the second day and onwards) thus does not represent the normal formation of bile acids in the rat but rather the maximal capacity of the liver for bile acid production. This capacity is fully made use of when bile acids are continually withdrawn in a fistula and the portal blood does not carry any bile acids back to the liver. In Fig. 3 the normal synthesis of taurocholic acid compared to the large synthesis which occurs after preformed bile acids have left the enterohepatic circulation is shown. The amount of bile excreted in the bile fistula rat is thus increased 10-20 fold, as a result of the broken enterohepatic circulation.

Our values for excretion of cholesterol in bile agree with those found by Friedman and Byers(5). In spite of the large synthesis of bile acids in fistula animals serum cholesterol remained unaffected. Apparently, the effects of these metabolic changes in cholesterol-bile acid metabolism are mainly limited to the liver in these experiments.

*Summary.* 1. A detailed analysis of excretion of taurocholic taurochenodesoxycholic acid and cholesterol in bile duct cannulated rats has been undertaken. 2. A constant excretion pattern consisting of 2 parts

was observed: A) Excretion of preformed bile acids present in the enterohepatic circulation during 12 hours following cannulation; B) An increased formation of bile acids resulting in a 10-20 fold increased synthesis of bile acids (40-70 mg/day), as compared to synthesis of bile acids in the intact animal (3-4 mg/day), is present from the second or third day.

The author is indebted to Professor S. Bergström and Dr. J. Sjövall for advice and facilities put at my disposal.

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Received January 23, 1957. P.S.E.B.M., 1957, v94.

## Influence of Thyroid Activity on Excretion of Bile Acids and Cholesterol in the Rat.*† (23019)

STEN ERIKSSON. (Introduced by O. Wintersteiner)
*Department of Physiological Chemistry, University of Lund, Sweden.*

It has long been realized that a relationship exists between plasma cholesterol concentration and thyroid activity(1). In general, hyperthyroidism is associated with decreased plasma cholesterol level and hypothyroidism with elevated concentration. Rosenman, Friedman and Byers(2) found biliary cholesterol in rats, increased in hyperthyroidism and decreased in hypothyroidism. On the basis of turn-over studies, Rosenman *et al.* (3) also concluded that thyroid hormone enhances cholesterol destruction. Marx, Gustin and Levi(4) measuring incorporation of $D_2O$ into cholesterol found cholesterol synthesis increased in thyroxine-injected and decreased in thyroidectomized rats. In recent years the direct importance of bile acid metabolism for quantitative aspects of metabolism of cholesterol has been stressed. Thus, work by Siperstein *et al.*(5) and by Bergström and Norman(6) has indicated that bile acids are the main metabolic end product of cholesterol. Thompson and Vars(7) studied the effects of altered thyroid activity on excretion of cholic acid and cholesterol in bile fistula rats. They expected cholic acid output to be high in the hyperthyroid state and low in the hyperthyroid state. However, they found lower values of cholic acid both in the hyperthyroid and the hypothyroid group as compared to control animals. In their experiments they did not determine taurochenodesoxycholic acid, the second bile acid of quantitative importance in the rat (Bergström and Sjövall(8)).

We have recently investigated excretion of cholesterol, taurocholic and taurochenodesoxycholic acid in normal bile fistula rats(9).

* Preliminary report was read at meeting of Norwegian Biochem. Soc., Oslo, June 4, 1956.

† This work has been supported by grant from the Medical Faculty, University of Lund.

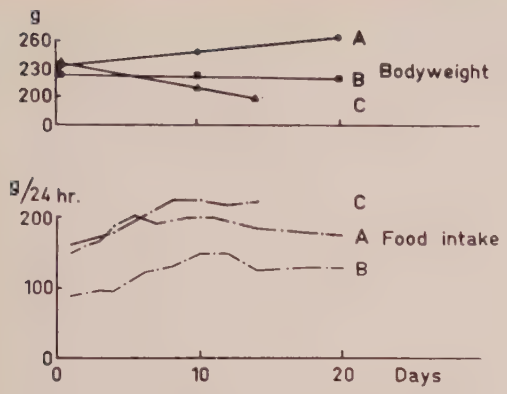


FIG. 1. Total food intake and avg body wt of A, 7 control animals, B, 7 hypothyroid animals and C, 7 hyperthyroid animals.

Using the same technic we have now investigated the influence of thyroid hormone on excretion of these compounds in the bile fistula rat.

*Material and methods.* White male rats of the institute stock (wt 210-270 g) were fed a synthetic diet consisting of a mixture of concentrate of pig feed and barley meal in proportions 2:1. Hyperthyroid rats were prepared by giving thyroid (Pulv. thyr. sicc. Iodine content 0.225%, AB Leo, Sweden) as .4% of diet for 10 days prior to operation, and after operation. Hypothyroid animals were prepared by giving propylthiouracil (Tiotil, Pharmacia, Sweden) as 0.5% of diet for 3 weeks prior to operation, and after operation. Euthyroid control animals were given the diet without any additions. The common bile duct was cannulated as previously described. The animals were given 0.9% NaCl to drink *ad libitum* during the experiment. Taurocholic and taurochenodesoxycholic acid were determined as previously described(9) using paper chromatographic separation as described by Sjövall. Bile cholesterol was determined as described previously(9).

*Results.* In Fig. 1 average body weight and food intake of animals in 3 experimental groups are presented graphically. The hyperthyroid rats failed to gain weight in spite of increased food intake. These animals further exhibited such clinical signs of thyrotoxicosis as increased pulse rate and tremors. The hypothyroid rats did not show any weight loss in spite of diminished food intake as compared to controls.

In Fig. 2 the excretion pattern of bile acids in the various groups is presented. In euthyroid animals, total excretion and proportion between taurocholic and taurochenodesoxycholic acids were the same as when the diet consists of oats and white bread(9). In the euthyroid group approximately 15% of total bile acid output is taurochenodesoxycholic acid. The gradual fall in bile acid excretion during the first day of collection is clearly demonstrated. As previously pointed out the amounts excreted during the first 6 or 12 hours represent bile acids present in the enterohepatic circulation. In the hypothyroid group the circulating amount of bile acids as judged from amount excreted during first 12 hours, is somewhat higher than in the control group. Less than 10% of this amount consists of chenodesoxycholic acid. In the hyperthyroid group the circulating bile acid pool is clearly elevated. Thus excretion of bile acids during the first 12 hours is almost twice that found in control animals. Approximately 50% of the amount excreted is chenodesoxycholic acid. Total excretion of bile acids during subsequent days in the hypothyroid group is considerably decreased. As previously pointed out, the plateau level reached on second or third day after operation represents maximal capacity of the liver for bile acid production. In the hypothyroid state this capacity thus seems to be impaired

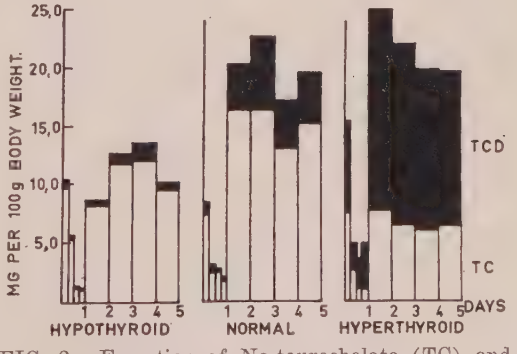


FIG. 2. Excretion of Na-taurocholate (TC) and Na-taurochenodesoxycholate (TCD) in the 3 experimental groups. Values are expressed as mg/100 g body wt. Each column in the diagram represents mean value of 4 animals.

TABLE I. Influence of Thyroid Activity on Excretion of Cholesterol in Bile Fistula Rats.

| Hr after operation | Avg excretion of cholesterol (mg/100 g body wt) in rats | | |
|---|---|---|---|
| | 4 hypothyroid | 4 control | 4 hyperthyroid |
| 0- 6 | .15 | .14 | 1.60 |
| 6- 12 | .09 | .11 | 2.05 |
| 12- 18 | .01 | .04 | .52 |
| 18- 24 | .03 | .09 | .64 |
| 24- 48 | .36 | .93 | 3.44 |
| 48- 72 | .44 | .96 | 3.91 |
| 72- 96 | .45 | .90 | 3.70 |
| 96-120 | .31 | .81 | 2.63 |

to a considerable degree. In the hyperthyroid animals total amounts of bile acids excreted during the last 4 days of experiment are approximately as large as the corresponding amount in control animals.

From Fig. 2 it also is obvious that the level of thyroid activity has a pronounced influence upon composition of the bile acid mixture formed. In the hypothyroid rats the amount of chenodesoxycholic acid excreted is reduced to less than 10% of the total. On the other hand, in the hyperthyroid state there is a shift towards chenodesoxycholic acid which represents about 70% of total amount excreted as against 15% in normal rats. Thus the decrease of cholic acid output in these rats is fully compensated for by increased formation of chenodesoxycholic acid.

In Table I excretion of cholesterol in the various groups is given. It is seen that hyperthyroid rats excrete far more cholesterol than normal rats. This finding confirms the results obtained by Rosenman, Friedman and Byers(2).

There is a fall in excretion of cholesterol during the first day after operation (Table I). A minimum usually occurs 18-24 hours after operation. This minimum is followed by increased excretion of cholesterol and a plateau is reached on the second day. This minimum can be seen in all groups.

The mechanism behind the findings reported is obscure. It should be stressed that the bile fistula animals are in a highly unphysiological state and further work must be done to be able to compare these results with changes that occur in the intact animal.

*Summary.* The influence of thyroid activity upon excretion of bile acids in bile fistula rats has been studied. In the hypothyroid state formation of both cholic acid and chenodesoxycholic acid is diminished. In the hyperthyroid state excretion of bile acids is not decreased but the composition is changed in that taurocholic acid is markedly decreased but taurochenodesoxycholic acid increased.

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Received January 23, 1957. P.S.E.B.M., 1957, v94.

# Simultaneous Determination of Cortisol and Corticosterone in Human Plasma by Quantitative Paper Chromatography.* (23020)

PHILIP K. BONDY AND G. VIRGINIA UPTON
*Department of Medicine, Yale University School of Medicine, New Haven, Conn.*

Although there are several successful methods for determination of cortisol (hydrocortisone)(1,2) or 17-hydroxycorticosteroids(3,4) in plasma, methods proposed for determination of corticosterone are less satisfactory. Previously described technics have depended either on differential color reactions of only moderate specificity(5) or on use of columns for separation of a "cortisone-like" material (1,6). Application of quantitative paper chromatography to the determination of plasma cortisol in this laboratory(2) suggested that similar methods might be used for determination of corticosterone. A method has been developed for simultaneous determination of cortisol and corticosterone in the same plasma sample.

*Procedure.* Blood is drawn into heparinized syringes and centrifuged within 15 minutes. A 1 ml portion of a standard ethanolic solution of cortisol-4-$C^{14}$ and corticosterone-4-$C^{14}$ containing 800 counts/minute of each compound is pipetted into 60 ml centrifuge tube and evaporated to dryness. With radioactive steroids now available, this represents about 0.2 $\mu$g of each substance. Twenty ml of plasma are added, followed by 0.5 ml of 1 N NaOH. The mixture is stirred and then extracted promptly three times with 20 ml of freshly redistilled chloroform. The pooled chloroform extracts are transferred to 60 ml boiling flask with a small well in the bottom(3) and evaporated to dryness at room temperature. The sides of the flask are washed into the well several times with 1-2 ml of chloroform. Contents of well are then transferred quantitatively with chloroform to a small area at origin of strip of Whatman No. 1 filter paper, previously extracted in Nolan extractor for 72 hours with boiling methanol. A standard spot (approximately 25 $\mu$g each of cortisol and corticosterone) is placed on the same line. Spots are placed 3 cm apart, except for the standard, which is placed at greater distance from the sample to avoid overlapping as the spot spreads during chromatography. Chromatography is performed in 2 stages. For the initial step, the paper is inserted in the trough with spots in the dependent position and after at least 2 hours' equilibration in an atmosphere of petroleum ether saturated with 75% methanol, the mobile phase (petroleum ether saturated with 75% methanol) is added to the trough and permitted to wash over the spots and off the paper, which required approximately 4 hours. The paper is then removed from the tank, air dried and reinserted with the steroid spots upward in another tank, equilibrated overnight and developed for 3 hours with toluene saturated with 70% methanol. The paper is allowed to dry and the strip containing the standard spot is then cut off and steroids located with ultraviolet handlamp. With this as a guide, 3 x 4 cm rectangles (for cortisol) and 4 x 7 cm rectangles (for corticosterone) are cut out. Since the spots sometimes tend to streak in direction of flow, the rectangle should be displaced slightly toward the solvent front rather than centered exactly on the standard spot. A blank is provided by cutting out a similar rectangle between cortisol and corticosterone spots. The steroids are eluted from paper by soaking in 4 ml absolute ethanol in test tubes sealed with aluminum-foil-covered corks for one hour at 40°C. The eluate is shaken and then 0.5 ml is taken for estimation of radioactivity and aliquots of the remainder are taken for fluorometric analysis. For alkaline fluorescence, duplicate 0.8 ml portions are pipetted into fluorometer cuvettes, dried *in vacuo,* and read one hour after addition of 0.5 ml of 0.3 N potassium tert butoxide, using 5860 Corning filter in the ex-

* Supported by research grant from Nat. Inst. of Arthritis and Metabolic Diseases of N.I.H., U. S. Public Health Service.

TABLE I. Recovery of Corticosterone.

| No. of exp. | Initial plasma conc., μg/100 ml | Corticosterone added, μg/100 ml | Recovery (%) | | |
|---|---|---|---|---|---|
| | | | Chemical | Radioactive | Corrected |
| 4 | .29 | 13.8 | 52.3 ± 7.0 | 78.3 ± 6.4 | 82.1 ± 4.1 |
| 6 | .00 | 9.8 | 72.3 ± 3.0 | 81.3 ± 5.8 | 88.8 ± 6.0 |
| 3 | .38 | 9.8 | 79.9 ± 4.2 | 65.6 ± 2.7 | 115.7 ± 4.3 |
| 3 | .29 | 9.8 | 74.8 ± 2.8 | 66.8 ± 5.3 | 106.3 ± 4.4 |
| 9 | .00 | 9.8 | 61.2 ± 8.3 | 73.1 ± 9.7 | 82.6 ± 9.9 |
| 25 | — | — | 67.9 ± 9.2 | 74.3 ± 7.2 | 90.9 ±13.4 |

Corticosterone was added to plasma immediately before extraction.

citing beam and 2418 Corning filter for emitted light(7). For sulfuric acid fluorescence, two 0.3 ml portions are pipetted into fluorometer cuvettes, 0.7 ml of concentrated sulfuric acid is added to each fluorometer tube, and fluorescence is read after one hour in Farrand fluorometer, using 3389 and 5113 Corning filters in the exciting beam and 3486 Corning filters for the emitted light(8). Both types of fluorescent measurements can be made on each sample if necessary. For each steroid a standard curve is also determined, using 0.5, 1 and 2 μg of cortisol, and 0.05, 0.1 and 0.5 μg of corticosterone. The 0.5 ml aliquots intended for radioactive measurements are pipetted into 30 ml optically clear vial and the ethanol evaporated to dryness. The residue is then dissolved in 15 ml of scintillation mixture (0.4% 2, 5-diphenyl-oxazole and 0.03% 1,4di(2[5-phenyloxazole]) benzene in toluene) and placed in a liquid scintillation counter (Technical Measurements Corp. Model LP2A) where it is counted for 5 minutes. The counter has an efficiency of 65% and a probable error of counting, under these circumstances, of 2%. With the quantities used, about 100 counts/minute are observed in the aliquot of paper eluate. Plasma concentration is calculated from the observed fluorescence after subtracting the paper blank. The figure is corrected for radioactive recovery, and the quantity of steroid added as radioactive tracer is subtracted from the total (2).

Corticosterone and cortisol are separated in a satisfactory manner by the second (toluene/70% methanol) chromatography; however, if this separation is performed without preliminary purification of the plasma extract, large amounts of spontaneously fluorescing material contaminate the area in which corticosterone is found. By use of preliminary chromatographic purification with petroleum ether/75% methanol, the fluorescent contaminant is washed off the paper without any alteration in position of either corticosterone or cortisol. The justification for introducing the radioactive correction has already been discussed(2). As a result of the use of this correction, added cortisol is completely recovered. Recovery of corticosterone is presented in Table I. Recovery is not quite so good as for cortisol(2), perhaps in part because cortisol travels a shorter distance and therefore the spot has less tendency to spread. In the original studies of plasma cortisol concentration, the steroid was determined by measuring its fluorescence in potassium butoxide(7). This method has the advantage of high specificity for compounds containing the 4-ene, 3-one configuration, but it is somewhat less sensitive than the sulfuric acid fluorescence described by Sweat(8). The blank fluorescence by Sweat's method also proved to be somewhat lower than with the alkaline reagent. A comparison was therefore made between the results obtained by the 2 methods, and no significant difference was found. Since maximum sensitivity is needed for determining minute quantities of corticosterone in human plasma, the sulfuric acid method may be preferable. The sensitivity of this method has been increased approximately 50% by reading the fluorescence in a 7:3 sulfuric acid:ethanol mixture rather than the 9:1 mixture recommended by Sweat(8). The altered sulfuric acid concentration does not modify characteristics of the excitation or

TABLE II. Cortisol and Corticosterone Concentrations in Plasma of Normal Subjects.

| | Cortisol | | | Corticosterone | | |
|---|---|---|---|---|---|---|
| | ♂ | ♀ | Total | ♂ | ♀ | Total |
| | μg/100 ml | | | μg/100 ml | | |
| No. | 16 | 13 | 29 | 16 | 13 | 29 |
| Mean | 11.4 | 8.8 | 10.2 | 1.8 | .8 | 1.3 |
| S.D. | 3.7 | 2.9 | 3.6 | 3.0 | 1.6 | 2.5 |
| Median | 11.6 | 9.0 | 10.3 | .6 | *.0 | .2 |
| Range | 5.9–17.7 | 4.0–13.1 | 4.0–17.7 | .0–4.0 | .0–5.0 | .0–5.0 |

* Failure to find corticosterone indicates that less than 0.1 μg/100 ml of plasma is present.

emission spectra from those originally described. Fluorescence reaches a stable plateau within an hour and remains stable for several hours thereafter.

*Observations.* When the technic was applied to the study of plasma from normal volunteers, the concentration of corticosterone was very low (Table II). The cortisol concentration is not significantly different from that previously reported(2). Although distribution of cortisol is approximately normal, the large discrepancy between mean and median of the corticosterone concentration emphasizes the significant skewing of distribution.

Plasma cortisol and corticosterone concentrations were measured in several patients convalescent from diseases not primarily affecting the adrenal glands during stimulation with adrenocorticotrophic hormone. The pituitary hormone was administered intravenously at a steady rate of 5 units/hour for 5 hours, and plasma drawn for analysis just before and at termination of the infusion. The results are seen in Table III. In every instance except one, the corticosterone concentration rose after ACTH.

TABLE III. Effect of ACTH on Plasma Cortisol and Corticosterone Concentrations, in 12 Subjects.

| Cortisol, μg/100 ml | | Corticosterone, μg/100 ml | |
|---|---|---|---|
| Initial | Final | Initial | Final |
| 11.6 | 24.1 | *.0 | .1 |
| 21.2 | 46.8 | .0 | 2.4 |
| 32.0 | 56.3 | .0 | .0 |
| 14.8 | 39.9 | 1.3 | 8.7 |
| 14.5 | 50.1 | .7 | 9.7 |
| 18.7 | 32.0 | .5 | 5.3 |
| 7.8 | 28.8 | .1 | 4.8 |
| 14.1 | 30.1 | 2.8 | 8.6 |
| 10.2 | 39.4 | 1.4 | 23.4 |
| 15.3 | 39.5 | .9 | 7.7 |
| 19.3 | 32.5 | 2.5 | 11.9 |
| 17.5 | 40.3 | .0 | 6.0 |

* See footnote to Table II.

*Discussion.* Corticosterone has been isolated and identified in plasma from the human adrenal vein(1,9-11) and breakdown products of the steroid have been found in human urine(12). Presumably, therefore, appreciable amounts of this substance are present in the peripheral plasma of man. Previous studies(1,5-7) have reported widely varying quantities of steroid, partly perhaps because of inadequate purification of the plasma extract before measurement of the hormone, and perhaps also in part because of unpredictable variability of recovery of the steroid. The present method utilizes a paper chromatography system of high resolution for purification and corrects for losses incurred during analysis by radioactive controls of each determination.

The identity of the steroids measured as "cortisol" and "corticosterone" can not be established absolutely. In regard to the former, however, the chromatographic characteristics combined with fluorescence in alkali indicate that it is a $\Delta^4$, 3-ketosteroid of characteristics similar to cortisol or cortisone. The fact that fluorescence in sulfuric acid is equal to that in alkali, eliminates cortisone as a quantitatively important component of this spot. The "corticosterone" spot, similarly, might also include 11-dehydrocortisone, but the correspondence between alkaline and acid fluorescence indicates that no significant quantities of the 11-ketonic steroid are present. In view of the definite identification of cortisol and corticosterone in human adrenal vein blood, and the failure to find appreciable quantities of other $\Delta^4$, 3-ketosteroids which might be difficult to separate in the chroma-

TABLE IV. Variability of Cortisol/Corticosterone Ratio.

| Subject | Date | Cortisol, μg/100 ml | Corticosterone, μg/100 ml | Cortisol / Corticosterone | Remarks |
|---|---|---|---|---|---|
| B. | 9/21/56 | 8.4 | 10.4 | .8 | Upper resp. infection |
| | 11/ 9 | 11.6 | .8 | 14.5 | Normal |
| R. | 9/21 | 19.2 | 19.4 | 1.0 | 48 hr post-dental extr. |
| | 11/ 2 | 13.1 | .0 | *(131) | Normal |
| | 11/ 9 | 14.6 | 1.2 | 12.7 | Acute urticaria |
| J. | 10/ 8 | 18.7 | 1.0 | 18.7 | |
| | 10/28 | 21.2 | .0 | *(212) | 1 wk on depot ACTH |
| | 11/ 5 | 13.1 | .6 | 21.8 | 2 " " " " |

* This ratio was computed assuming that a corticosterone conc. of .0 actually represents a conc. of .1 μg/100 ml.

tographic system used for this analysis, it seems highly probable that the substances being studied are indeed cortisol and corticosterone.

Bush(13,14) has recently reviewed information regarding the relative concentrations of cortisol and corticosterone in human plasma. The range of cortisol/corticosterone reported by various observers ranges from 0.9 to 10.0. In our observations the mean ratio was 7.9; however, in the many instances where no corticosterone could be detected, the ratio is meaningless. Although the ratio did not change much in patients receiving ACTH, it was not constant in any given individual whose plasma was analyzed on several occasions (Table IV). This seems to speak against the hypothesis advanced by Bush that the ratio is an inherent quality of the individual and suggests that the pattern of secretion may be altered by the environment. Recent observations(15,16) that corticosterone may be cleared from the plasma much more rapidly than cortisol suggest that the plasma concentrations may not necessarily reflect rates of secretion of the 2 substances by the adrenal, but may be the resultant of the respective rates of secretion and removal.

*Summary and conclusions.* A method is presented for measuring cortisol and corticosterone simultaneously in a single specimen of plasma. The steroids are purified by paper chromatography, eluted and measured by fluorometry. Losses incurred during analytical procedure are compensated by application of an isotope dilution correction. The normal range of corticosterone in human beings is 0 to 5 μg/100 ml, with a mean of 1.3 ± 2.5 μg/100 ml. The normal range for cortisol is 4.0 to 17.7 μg/100 ml, with a mean of 10.2 ± 3.6 μg/100 ml.

The authors are grateful to Dr. S. R. Lipsky for help in designing the isotope experiments and to Dr. P. Mandelstam who assisted in some of the ACTH tests. The cortisol-4-$C^{14}$ and corticosterone-4-$C^{14}$ were kindly donated by the N.I.H. Nonradioactive steroids were kindly donated by Merck and Co. and the Upjohn Co.

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Received January 23, 1957. P.S.E.B.M., 1957, v94.

## Insulinase-Inhibitory Activity of Protein Hydrolysates.*† (23021)

I. ARTHUR MIRSKY AND GLADYS PERISUTTI

*Department of Clinical Science, University of Pittsburgh School of Medicine.*

During studies of the identity of the factor (or factors) in liver extracts which inhibits activity of insulinase(1), it became apparent that the greatest concentration of this factor occurred in fractions reported by others to be rich in strepogenin activity(2). Since hydrolysates of insulin and a variety of other proteins are also rich in strepogenin activity (2,3), the insulinase-inhibitory activity of such hydrolysates was studied.

*Methods.* The various proteins used were hydrolyzed for 3 hours with concentrated hydrochloric acid (4 g protein/100 ml acid) at 37°C, neutralized and evaporated to dryness in flash evaporator. The hydrolysates were kept in the dry state until immediately before use.¶ The effect of the presence of various concentrations of the protein hydrolysates on rate of degradation of $I^{131}$ labeled insulin by liver extract was determined by the procedure described previously(4). Inhibitory activity of the 3-hour acid hydrolysates of proteins was computed on the basis of quantity of nitrogen necessary to produce a 50% inhibition of insulinase activity of the extract. Since an hydrolysate of insulin had the greatest inhibitory activity, the activities of other hydrolysates were expressed as percentage of activity of the insulin hydrolysate. The effect of prolonged acid hydrolysis on insulinase-inhibitory activity of crystalline insulin, bovine plasma albumin and casein was determined by treating these proteins as above, with concentrated hydrochloric acid for 72 hours at 37°C. Aliquots were removed at the beginning and at various designated intervals during hydrolysis, neutralized and lyophilized. The quantity of each hydrolysate, in terms of nitrogen, necessary to produce a 50% inhibition of insulinase activity of a liver extract was determined. Maximum insulinase-inhibitory activity of an hydrolysate was attributed to that aliquot which produced a 50% inhibition with the smallest quantity. All other assays were expressed in terms of percentage of aliquot with maximum activity. In addition, tryptic and chymotryptic digests of insulin, bovine plasma albumin, crystalline lysozyme and casein were prepared. The proteins were dissolved or dispersed in distilled water, heated at 100°C for 3 minutes, cooled and adjusted to pH 7.8. A 0.5% solution of each protein was incubated under toluene for 12 hours at 37°C with an amount of crystalline trypsin or crystalline chymotrypsin equal to 1% of weight of the protein. pH was maintained during incubation period by addition of alkali. At the end of incubation period, the reaction mixtures were adjusted to pH 3, heated to 100°C, cooled, neutralized and lyophilized. The action of hydrolysates on degradation of $I^{131}$ labeled insulin was determined as above using the equivalent of 5 mg of protein. The data were expressed in terms of activity of an equivalent amount of a 3-hour acid hydrolysate of crystalline insulin. The type of inhibition produced by some of the hydrolysate of proteins was determined from studies of the effect of single concentration of each hydrolysate on action of

* Aided by grants from Eli Lilly Co., and Foundations' Fund for Research in Psychiatry.

† Presented in part at Lilly Insulin Conference, Indianapolis, May 1955.

¶ We are indebted to the Lilly Research Laboratories for some of the hydrolysates used in this study.

TABLE I. Insulinase-Inhibitory Activity of 3-Hour Acid Hydrolysates of Various Proteins. Inhibitory activity computed on basis of quantity of hydrolysate (in terms of N) required to produce 50% inhibition of insulinase activity of liver extract and expressed in terms of activity of an hydrolysate of insulin.

| Protein hydrolysate | % activity |
|---|---|
| Insulin, crystalline, Lilly No. 535664 | 100.0 |
| Pitressin, Parke-Davis, No. 174363 | 87.1 |
| Pitocin, Parke-Davis, Nos. 182530 and 182531 | 83.0 |
| Growth hormone (Somar), Armour, No. M-208 | 81.3 |
| Glucagon, crude, Lilly, E-1399 | 77.3 |
| Intrinsic factor, Lilly, No. 260-201B-51-3 | 72.7 |
| Adrenocorticotrophin, Armour, No. 41-L-2 | 57.8 |
| Prolactin, crude, 6 units/mg, Lilly, No. 953561 | 42.0 |
| Lysozyme, crystalline, Armour, No. 00325 | 42.0 |
| Bovine plasma albumin, Fraction V. Pentex, No. B6004 | 38.1 |
| Trypsin, crystalline, Pentex, No. A3401 | 38.1 |
| Alpha Amylase, Nutritional Biochemical, No. 2796 | 38.1 |
| Egg albumin, crystalline, Armour No. E90115, Pentex No. B4902 | 37.8 |
| Casein, vitamin-free, Nutritional Biochemical | 36.4 |
| Serum albumin, human, Lilly, No. 352319B | 33.4 |
| Renin substrate, Lilly, No. 235-195B-104 | 33.4 |
| Chymotrypsin, crystalline, salt-free, Pentex, No. A2701 | 33.4 |
| Serum albumin, bovine, crystalline, Pentex, No. A1201 | 32.2 |
| Globin, purified, Nutritional Biochemical | 30.8 |
| Catalase, port liver, Pentex, No. A3901 | 30.0 |
| Chymotrypsin, crystalline, Bios | 29.8 |
| Pepsin, 3× crystallized, Pentex, No. C3705 | 29.0 |
| Ribonuclease, crystalline, salt-free, Pentex | 28.3 |
| Beta-Lactoglobulin, crystalline, Pentex, No. A4801 | 27.6 |
| Gamma globulin, bovine, Pentex, No. C0602 | 25.8 |
| Tetanus antitoxin, Lilly, No. B-8652B | 25.8 |
| Renin, porcine, purified, Lilly, No. 221-194B-213 | 25.5 |
| Malt diastase, Nutritional Biochemical, analytical grade | 24.3 |
| Plasma albumin, bovine, crystalline, Armour, No. M66605 | 24.0 |
| Fibrinogen, bovine, Armour, No. N1804 | 24.0 |
| Protamine, (Salmine), Lilly, No. 961271 | 23.4 |
| Gamma globulin, porcine, Pentex, No. A2001 | 23.2 |
| Egg albumin, 5× crystallized, Pentex, No. B4903 | 21.7 |
| Emulsin, Nutritional Biochemical, No. 6076 | 21.6 |
| Trypsinogen, crystalline, General Biochemicals, No. 28910 | 20.5 |
| Papain, Lilly, No. 221-267B-31 | 20.2 |
| Ficin, General Biochemicals, No. 20142 | 17.3 |
| Immune serum globulin, human, Lilly, No. 8471X342644Z | 16.6 |

extract incubated with different concentrations of labeled insulin. The effect of an acid hydrolysate of bovine plasma albumin on destruction of $I^{131}$ insulin by intact mice was determined by procedure described previously(5).

*Results.* It is apparent from the data summarized in Table I that a fairly large number of proteins yield insulinase-inhibitory fragments after relatively mild acid hydrolysis. The hydrolysates of 12 other proteins that were studied had no inhibitory activity.‡ A Lineweaver-Burk(6) plot of the effect of hydrolysates of crystalline insulin and bovine plasma albumin on destruction of various concentrations of labeled insulin by fresh liver extract suggests that inhibition produced by various hydrolysates is of the competitive type (Fig. 1).

The peptide nature of fragment (or fragments) responsible for insulinase-inhibitory activity of the protein hydrolysates is indicated by complete loss of inhibitory activity which occurs after prolonged hydrolysis of the proteins. The data illustrated in Fig. 2 reveal that hydrolysis of insulin, bovine plasma albumin and casein with concentrated hydrochloric acid results in maximum inhibitory activity in 2 to 6 hours; thereafter insulinase-inhibitory activity diminishes so that

‡ The 12 proteins were crystalline phosphorylase, crystalline trypsin, edestin, ovamucoid, urease, glucuronidase, hemoglobin, trypsin-inhibitor, protamine sulfate, alpha amylase, beta amylase, and elastin.

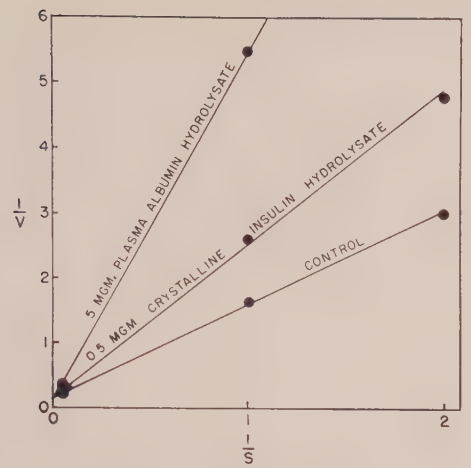


FIG. 1. Inhibition of insulinase activity of rat liver extract by acid hydrolysates of crystalline insulin and bovine plasma albumin. Incubation of 1 ml of liver extract plus 1 ml of phosphate buffer containing various quantities of $I^{131}$ insulin or plus 1 ml phosphate buffer containing designated amounts of protein hydrolysate and various quantities of insulin, at pH 7.8 and 37°C for 30 min. V = units of insulin destroyed in 30 min. S = units of insulin/ml of incubation mixture.

after 48 hours of hydrolysis there is essentially no activity.

Acid hydrolysates of proteins, which are effective as insulinase-inhibitors *in vitro* may also decrease destruction of insulin by the intact animal. Thus, subcutaneous administration of 4 mg of an hydrolysate of bovine plasma albumin/g body weight to 24 mice resulted in reduction of rate of insulin destruction from 15.8 ± 0.5 to 10.5 ± 1.1 units/100 g/hour ($P<0.001$). Administration of such hydrolysates to mice or rats, however, does not produce a decrease in blood sugar concentration.

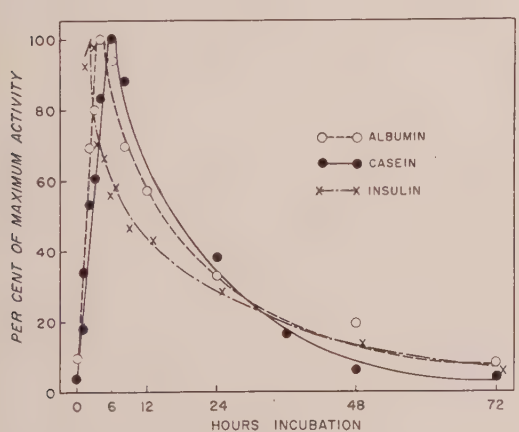


FIG. 2. Effect of prolonged acid hydrolysis of crystalline insulin, bovine plasma albumin and casein on insulinase-inhibitory activity. (See text.)

Enzymatic digests of various proteins are effective as inhibitors of the destruction of insulin by potent extracts of liver (Table II). Thus, incubation of crystalline insulin, casein, albumin and lysozyme with crystalline trypsin and crystalline chymotrypsin at 37°C and pH 7.8 for 12 hours yields products which inhibit insulinase (Table II). It is interesting that whereas a tryptic digest of bovine plasma albumin is more active than a chymotryptic digest in inhibition of insulinase, a chymotryptic digest of crystalline lysozyme is more active than a tryptic digest.

TABLE II. Insulinase-Inhibitory Activity of Tryptic and Chymotryptic Digests of Proteins Expressed in Terms of Inhibition Produced by Equivalent Amount of 3-Hour Acid Hydrolysate of Crystalline Insulin.

| Protein | Inhibition (%) | | |
|---|---|---|---|
| | Untreated | Tryptic digest | Chymotryptic digest |
| Crystalline insulin | 100.3* | 98.1 | 102.1 |
| Bovine plasma albumin | 19.0 | 52.0 | 19.8 |
| Crystalline lysozyme | 18.1 | 20.8 | 106.0 |
| Casein | 7.5 | 48.6 | 57.3 |

* Reflects dilution of labeled insulin.

*Discussion.* Our studies reveal that an acid or an enzymatic hydrolysis of various proteins yields peptides which can competitively inhibit the action of insulinase *in vitro* and *in vivo*. The inability of these hydrolysates to lower blood sugar concentration of mice and rats may be due to the presence of glucogenic as well as insulinase-inhibitory peptides. Although the hydrolysates which exhibit greatest insulinase-inhibitory activity are also those which have the greatest strepogenin activity(2,3), the 2 activities are not related. Thus, purified polypeptides§ which have a fair degree of strepogenin activity, exhibit no insulinase-inhibitory activity.

§ We are indebted to Dr. W. Woolley for providing samples of serylhistidylleucylvalylglutamic acid and serylhistidylleucylvalylglutamylalanylleucine.

The nature of peptides responsible for inhibition of insulinase remains unknown. In view of the observation that a variety of natural and synthetic plant growth regulators exert an insulinase-inhibitory and hypoglycemic action(7,8), it is interesting to note that enzymatic digests of plant and animal proteins are rich in auxins which apparently are newly formed during protein digestion(9, 10). Whether or not auxin activity and insulinase-inhibitory activity of protein hydrolysates are related awaits further study.

Tomizawa and Williams have reported that addition of casein, $\alpha$-corticotrophin trichloracetate, glucagon and growth hormone to the incubation mixture of liver extract and labeled insulin results in decrease in rate of insulin destruction. They interpret this observation to mean that added proteins compete with insulin for a non-specific enzyme system(11). The data reported herein do not support the conclusion of Tomizawa and Williams. Thus, acid hydrolysates of glucagon, corticotrophin, growth hormone and casein are among the most potent inhibitors of insulinase. These proteins are also degraded by extracts of liver which hydrolyze insulin(12). Consequently, it is probable that inhibition of insulin destruction which occurs *in vitro* in the presence of the above proteins is due to release of insulinase-inhibitory peptides rather than to competition between insulin and any of the aforementioned proteins.

*Conclusion.* Destruction of insulin by extracts of rat liver is inhibited competitively by 3-hour acid hydrolysates and by tryptic and chymotryptic digests of a variety of proteins. Insulinase-inhibitory activity is attributed to a peptide (or peptides) which loses activity on complete hydrolysis.

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Received January 24, 1957. P.S.E.B.M., 1957, v94.

# Contribution to Understanding of Mechanism of Permissive Action of Corticoids. (23022)

FRANK L. ENGEL* AND JOAN FREDERICKS
*Departments of Medicine and Physiology, Duke University, Durham, N. C.*

The mechanism of interaction in metabolism between corticoids and response to injury, has been the subject of considerable debate in recent years(1,2,3). One issue has been whether corticoids should be considered as "prime movers" in the response or whether their role is important but basically secondary one. The first point of view is expressed clearly in the following interpretation of the hyperglycemic response to trauma written by Selye and Horava in 1953(4), and describing the results of an earlier experiment of Selye and Dosne(5). "Traumatic shock produces hyperglycemia in the intact, but hypoglycemia in the adrenalectomized rat. If an adrenalectomized rat is maintained on threshold doses of adrenocortical extracts (which in themselves cause no hyperglycemia), it can again respond to surgical shock normally, that is by a marked rise in blood sugar. Apparently the stress of traumatic injury does not merely raise the blood sugar through excessive production of glucocorticoids, nor do the glucocorticoids exert their maximal hyperglycemia action by themselves. We may conclude that the metabolic changes incident to stress produce favorable conditions for the manifestation of the hyperglycemic effect of the glucocorticoids." Although Selye qualifies this interpretation by writing " . . . it is immaterial whether we say that the hormone conditions the effect of the non-hormonal stimulus, or vice versa, since synergism between both factors is necessary to obtain an optimal response," it is still of considerable importance for the interpretation of the theory of diseases of adaptation to establish the order of causality, if possible. This is particularly true since Selye has written elsewhere(1), "Stress itself is perhaps the most effective and most common factor capable of conditioning the action of adaptive hormones." If trauma conditions the action of the glucocorticoids then the hormone must be considered the cause of the response. Extending this to Selye's concept of the diseases of adaptation the hormones become the chief mediators in some of these diseases which in turn must be considered as examples of hypercorticalism. The alternate point of view, championed by Ingle(2) and favored by ourselves(3), is that hormones play a supporting role in the reactions to trauma. In these terms the corticoids make conditions suitable for a normal response to trauma and as such presumably are tending to subserve homeostasis. The role of hormones in the etiology of diseases would then be considered as secondary to other factors. Thus, they might provide conditions suitable for development or manifestation of disease or might accentuate the signs or symptoms of disease but not themselves cause the disease. The activation of a peptic ulcer by glucocorticoids(6) may be an example of the first mechanism while accentuation of edema by aldosterone in nephrosis and congestive heart failure may be an example of the second. Since it has been shown that hyperglycemia of trauma depends chiefly on hepatic glycogenolysis(7), it has always seemed more reasonable to us to assume that the absence of this hyperglycemic response to trauma in the adrenalectomized rat relates in part at least to deficient stores of liver glycogen in the fasted adrenalectomized rat. If so, administration of cortical extract in Selye and Dosne's experiment would permit hyperglycemia simply by promoting gluconeogenesis and glycogen formation, thereby providing an hepatic source of glucose. If this interpretation is correct, one would anticipate that the fed adrenalectomized rat would exhibit an hyperglycemic response to trauma since it is well known that this preparation has adequate liver glycogen stores.

* Supported by research grant from Am. Cancer Soc., the Nat. Research Council, Office of the Surgeon General, Department of Army.

TABLE I. Influence of Trauma on Blood Glucose-Adrenalectomized Rats.

| Experimental groups | No. of rats | Blood glucose, mg % (mean ± S.E.) 0 hr | 1 hr | 2 hr | 3 hr |
|---|---|---|---|---|---|
| Control | | | | | |
| 1. Adrx.–fasted–saline–DOCA | 6 | 51 ± 3.7 | 57 ± 6.6 | 58 ± 4.3 | 51 ± 5.3 |
| 2. " –fed–saline–glucose–DOCA | 5 | 110 ± 12.0 | 104 ± 8.7 | 96 ± 5.0 | 85 ± 4.8 |
| 3. " –fasted–saline–DOCA–cortisone | 5 | 119 ± 10.6 | 108 ± 6.2 | 103 ± 7.5 | 92 ± 8.7 |
| Traumatized | | | | | |
| 4. Normal–fasted | 6 | 79 ± 4.4 | 157 ± 12.4 | 153 ± 15.8 | 142 ± 14.2 |
| 5. Adrx.–fasted–saline–DOCA | 11 | 62 ± 4.7 | 69 ± 5.2 | 59 ± 8.6 | 55 ± 8.6 |
| 6. " –fed–saline–glucose–DOCA | 6 | 117 ± 4.5 | 151 ± 10.8 | 131 ± 13.1 | 125 ± 14.8 |
| 7. " –fasted–saline–DOCA–cortisone | 6 | 69 ± 3.2 | 105 ± 7.8 | 90 ± 7.6 | 82 ± 11.5 |

The present experiment demonstrates the validity of this hypothesis.

*Method and materials.* Male albino rats of the Vanderbilt strain, weighing between 150 and 250 g were used. The animals were anesthetized with pentobarbital and bilaterally adrenalectomized through lumbar incisions. The following groups were studied. (1) Normal rats, fasted 16 hours. (2) Adrenalectomized rats maintained with 0.9% saline in their drinking bottles and subcutaneous injections of 0.5 mg DOCA daily. They were fasted 16 hours before the experiment. (3) Adrenalectomized rats as in group (2) but allowed free access to Purina Chow and 2.5% glucose in 0.9% saline until the time of trauma. To insure adequate liver glycogen stores, the animals were tube-fed 5 ml of a high carbohydrate liquid diet(8), 5 hours before trauma. (4) Adrenalectomized rats maintained with 0.5 mg DOCA and 1 mg cortisone acetate i. m. daily, 0.9% saline and fasted 16 hours. All animals were allowed to drink freely up to the time of trauma. The rats, anesthetized with pentobarbital, were traumatized 3-5 days after adrenalectomy in the same fashion as described by Selye and Dosne. *i.e.* by crushing the abdominal muscles, the stomach and cecum with a hemostat. Blood was drawn from the tail prior to trauma and 1, 2 and 3 hours thereafter, or at the corresponding times in the controls, and were analyzed for glucose by the method of Somogyi(9).

*Results.* The results are summarized in Table I. As expected, the fed and cortisone treated fasted adrenalectomized rats had significantly higher blood sugar levels than the fasted adrenalectomized animals during periods of observation without trauma. After trauma the normal fasted rat had an hyperglycemic response which the untreated fasted adrenalectomized animal did not share. In contrast to Selye and Dosne's results, however, the fasted adrenalectomized rats as a group did not develop significant hypoglycemia, although individual animals did. The difference in response is probably due to the fact that the rats in Selye and Dosne's experiments received only tap water to drink during the 16 hours fast, whereas our rats were protected somewhat against vascular collapse by DOCA and saline. The vascular collapse is a potent factor in causing hypoglycemia(7).

When the adrenalectomized rats were prefed carbohydrate, they exhibited blood sugar changes in response to trauma which were not significantly different from those of normal fasted traumatized rats. Finally, the adrenalectomized fasted group which had been pretreated with cortisone had an increase in blood sugar after injury which was of the same magnitude as the fed adrenalectomized animals, but was less sustained.

*Discussion.* These results demonstrate clearly that cortical hormone is not necessary for the hyperglycemic response to trauma, provided a source of glucose is present, presumably as liver glycogen. They make unnecessary Selye's assumption that trauma conditions the actions of the corticoids and hence that the hyperglycemia of trauma is a manifestation of hyper-

adrenalcorticism. In point of fact, they are more consistent with the reasoning used in Selye and Dosne's original report, in which the hyperglycemia was attributed to a continuing ability to resynthesize glucose from lactic acid in the corticoid treated rats, even though this is probably not the precise mechanism involved. They also conform better with Selye's earlier discussion in *Stress*(10) where the significance of glycogen stores for the hyperglycemic response to trauma is alluded to. In light of these considerations, Selye's more recent interpretation of these experiments is puzzling.

The demonstration that either the fed or the cortisone maintained fasted adrenalectomized rat reacts to trauma with hyperglycemia fits well with Ingle's "permissive" concept. In a real sense, cortical hormone may be considered as "permitting" the hyperglycemic response to trauma by making conditions metabolically favorable for a continuing supply of glycogen in the liver and consequently blood glucose. The same end is met by feeding. Obviously, in this example, neither adrenal hormone nor feeding *cause* the hyperglycemia of trauma, even though each by itself may raise the blood sugar if given in excess. Both simply provide the conditions which are necessary for the trauma to initiate an hyperglycemic response even though they do so through different primary mechanisms. This experiment illustrates in a graphic way what we understand by Ingle's "permissive" concept. The same formulation is unquestionably applicable to many other aspects of the adrenal-stress interaction, but not necessarily to all of them. The permissive concept does not imply an "all-or-none" effect as Selye(1) has repeatedly, but incorrectly, attempted to read into Ingle's use of the word "permissive" by the use of a light-switch analogy. As illustrated in the present report in some cases the hormone as well as other factors may "permit" the reaction to go on, while in other reactions the hormone may be essential and not replaceable by some other factor. Moreover, the magnitude of the response may be influenced by the hormone without it being necessary to assume that the hormone is the prime mover. Thus, in the present experiment, the dose of hormone would determine the level of the liver glycogen but the latter is reflected in only a modest elevation in blood sugar unless some other factor, such as trauma (as in this experiment), pancreatic insufficiency or something else enters the picture. The term "permissive" describes this type of biological interrelationship very well.

Selye has applied "conditioning" to this interaction but he has used and defined this term so variably in different publications that it now has a less specific meaning than "permissive" as used by Ingle and ourselves. Thus, within one year the following definitions of "conditioning" and "conditioning factors" may be found in Selye's writings: (1) "Conditioning factors are factors which have little or no activity but can significantly alter a response to a stimulus (*e.g.* sodium can act as a conditioning factor of DOCA activity)"(1). (2) Conditioning factors are "substances or circumstances which influence the response to an agent, for instance, a hormone."(11). (3) "Conditioning is a generic designation to include synergism, antagonism, potentiation, induction of reactivity where such would otherwise not exist (Ingle's original meaning of permissive)† and qualitative changes in reactivity"(12). These definitions are so broad that they tend to obscure rather than clarify our understanding of the mechanism of interaction of the hormones in the stress response.

We believe that the permissive concept as used by Ingle and defined and illustrated above, serves as a useful working basis not only for the understanding of the metabolic phenomena of stress just discussed, but also for the elucidation of the role of the hormones in certain pathological processes such as peptic ulcer, hypertension, inflammation, etc. We have discussed the application of this concept to these problems elsewhere(6,13). When more information is available concerning the mechanisms involved in each of the

† It should be noted that Ingle has never defined "permissive" in this manner. The above is Selye's misinterpretation of Ingle's use of the term.

reactions under consideration descriptive terms such as "permissive" and "conditioning" will have outlived their usefulness. Indeed, the continued use of these terms without acknowledgment of mechanisms when they are known serves to confound rather than clarify our understanding. An example of this may be found in a recent report(14) which refers to a high sodium intake as conditioning the action of desoxycorticosterone to cause muscular paralysis in dogs. Nowhere does the author acknowledge the well established facts that the paralysis is due to potassium depletion secondary to the influence of DOCA on renal excretion of potassium and that a high sodium intake accentuates potassium depletion by promoting renal loss of potassium.

*Summary.* The adrenalectomized rat may exhibit a normal hyperglycemic response to trauma if maintained with a constant dose of glucocorticoids while fasting or if untreated but fed. In both cases the normal response is interpreted as being dependent on an availability of liver glycogen to permit an hyperglycemic response while the failure of the fasted untreated adrenalectomized rat to develop hyperglycemia may be due to deficient glycogen stores rather than to hormone lack *per se*. The significance of these findings is discussed in relation to current concepts of the interaction of the adrenal cortex and the stress reaction.

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Received January 24, 1957. P.S.E.B.M., 1957, v94.

## Influence of Parathyroids on Removal of Citric Acid Administered by Peritoneal Lavage.* (23023)

JOSEPH R. ELLIOTT AND ROY V. TALMAGE
*The Rice Institute, Houston, Texas.*

Since Dickens(1) discovered that bone contains large quantities of citric acid, there has been much interest in the relationship of this acid to various bone constituents, and in its relation to over-all bone metabolism. Carlsson and Hollunger(2) have shown that citric acid may be produced in bone and reported an increased production of this acid following administration of vit. D. Elliott and Freeman(3,4) reported that citric acid changes parallel those of calcium in plasma of various animals and suggested that plasma increases in citric acid as well as those in calcium were probably supplied by bone. They further noted that either vit D or parathyroid hormone were able to raise plasma levels of both calcium and citrate. Neuman *et al.*(5) reported their belief that bone produces citric acid in response to parathyroid hormone which in turn is complexed with calcium and released into the plasma. It has also been shown(6) that endogenous citric acid, pro-

* This work was aided in part by grant by Atomic Energy Commission.

duced by blocking the citric acid cycle with fluoroacetate, increased plasma calcium in parathyroidectomized as well as in normal and nephrectomized rats. The evidence of the above reports suggests that citric acid takes an active part in bone metabolism, that citric acid and calcium are capable of forming complexes *in vivo,* and that citric acid may be able to mobilize calcium from the skeleton.

By use of the technic of continual peritoneal lavage, this laboratory has recently demonstrated the interrelation of calcium and endogenous citric acid in the control by the parathyroids of the equilibration between bone and extracellular calcium and citric acid levels(7). While both calcium and citric acid levels in the equilibrated peritoneal wash were reduced by parathyroidectomy in nephrectomized rats, the reduced levels remained fairly constant during the experimental period following parathyroidectomy. The purpose of the following series of experiments was to determine, by similar technics, the effect of exogenous citric acid on the calcium and citrate levels in normal, nephrectomized, and parathyroidectomized-nephrectomized rats, with the hope that these data would give further clarification of bone metabolism and the interrelation of citric acid and the parathyroid hormone.

*Methods and materials.* Male Sprague-Dawley rats weighing between 225-275 g were used in these experiments; however, for any one experiment the weight range was restricted to 25 g. For most experiments the animals were maintained on a calcium-free diet 3 days prior to use. Nephrectomy was performed through a ventral midline incision. At the same time, a peritoneal plug made from the specifications of Kolff and Page(8) was stitched into the incision. Parathyroidectomy was performed 24 hrs after nephrectomy. Parathyroids were removed individually. All operations were done under ether anesthesia. The peritoneal rinsing fluid was a calcium and phosphate-free isotonic fluid of a composition previously described(7), with the addition of varying amounts of citric acid and the subsequent adjustment of the rinse to a pH of 7.4. For the peritoneal lavage, 30 ml of rinsing fluid were placed in the peritoneal cavity and allowed to equilibrate for 1 hour before removal. Calcium and citric acid analyses were made on the wash after removal. Calcium determinations of the wash were made by precipitation with ammonium oxalate, redissolving in perchloric acid, and reading on a Beckman flame spectrophotometer. Citric acid determinations were made using the Hunter and Leloir modification(9) of the method by Pucher *et al.* (10).

*Results.* The data obtained in these studies are presented in 3 groups: 1) *Comparison of effect of exogenous citric acid in normal and nephrectomized rats*: Concentrations of citric acid of from .25 to .5% were added to calcium-free rinses administered to normal and 24 hr nephrectomized rats. Calcium and citric acid levels of the wash removed after one hour equilibration are given in Table I. The large part played by the kidney in the metabolism of citric acid is shown by comparison of normal and nephrectomized animals. It is obvious that in the normal animals much of the citric acid was removed by the kidney within the hour. The citric acid remaining in the wash of the nephrectomized animals was proportional to the amount added to the rinse, and for the first rinse appeared to approach a simple dilution with the extracellular fluid. In both the normal and the nephrectomized animal, the exogenous citric acid produced a marked rise in the calcium level of the wash. In each case the calcium level was a reflection of the wash level of citric acid rather than the administered level. Since the rinse used was calcium-free, this rise in calcium in the wash is indicative of the ability of exogenous citric acid to mobilize endogenous calcium.

2) *Comparison of effect of exogenous citric acid in nephrectomized and nephrectomized-parathyroidectomized rats*: Since the parathyroidectomized rats without kidneys were unable to survive a dosage of citric acid larger than 0.2%, this series of experiments was run using citric acid in the rinse in concentrations of 0.025 to 0.2%. The results are summarized in Table I. For comparative

TABLE I. Effect of Exogenous Citric Acid on Calcium and Citrate Content of First Peritoneal Wash.

| Citric acid in rinse* | Calculated by dilution‡ | Citric acid in wash† | | | Calcium in wash† | | |
|---|---|---|---|---|---|---|---|
| | | Normal | 24 hr Neph | 24 hr Neph 4 hr PTX | Normal | 24 hr Neph | 24 hr Neph 4 hr PTX |
| 0 | | 2.34 ± .43 (12) | 1.48± .36 (16) | .96± .27 (17) | | 7.08± .36 (16) | 3.49 ± .31 (17) |
| 25 | 10.9 | | 10.4 ± 2.6 (11) | 16.2 ±2.9 (11) | | 7.15± .45 (11) | 3.52 ± .32 (11) |
| 50 | 20.3 | | 19.4 ± 3.4 (12) | 28.8 ±3.2 (9) | | 8.32± .61 (12) | 3.72 ± .51 (9) |
| 100 | 39.1 | | 47 ± 4.8 (9) | 67.1 ±5 (12) | | 9.4 ± .72 (9) | 5.5 ± .42 (12) |
| 200 | 76.7 | | 80 ± 5.7 (4) | 89.6 ±4.6 (12) | | 12.3 ± .86 (4) | 5.7 ± .48 (12) |
| 250 | 95.5 | 84 ± 8 (6) | 96 ±11 (6) | | 12.2 ± 1.2 (6) | 13.8 ±1.4 (6) | |
| 400 | 151.9 | 89 ± 9 (6) | 138 ±22 (8) | | 12.4 ± 1.1 (6) | 15.6 ±1.7 (8) | |
| 430 | 161.8 | 94 ± 12 (8) | 152 ±19 (14) | | 12.7 ± 1.2 (8) | 16.8 ±2.1 (4) | |
| 500 | 189.5 | 98 ± 11 (7) | 193 ±26 (4) | | 12.5 ± 1.1 (7) | 17.3 ±2.6 (4) | |

* Rinse = Fluid placed in peritoneal cavity. † Wash = Fluid removed from peritoneal cavity after 1 hr equilibration. ‡ Extracellular fluid calculated as 20% body wt.

All values given in mg/100 ml. No. in parentheses are No. of animals in group. Neph = Bilateral nephrectomy. PTX = Parathyroidectomized. Values given with stand. error $\left( \text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}} \right)$.

purposes, an earlier experiment utilizing a citrate-free wash was extended. Rats parathyroidectomized approximately 24 hours after nephrectomy showed a small but significant drop in citric acid concentration of the wash after one hour equilibration, accompanied by the marked drop previously noted (5) in calcium concentrations. Following administration of citric acid it can be seen that the rise in the wash values for this ion is greater in the parathyroidectomized animal than in their nephrectomized controls. It must be assumed, therefore, that the parathyroids affect citric acid metabolism at a site other than the kidney. Despite the higher citric acid content of the wash, the calcium content of the wash from the parathyroidectomized rat was lower than its nephrectomized control, though a significant rise in calcium was noted. It would appear, therefore, parathyroidectomy affects the ability of citric acid to remove calcium from bone.

3) *Disappearance of exogenous citric acid administered in successive washes to normal, nephrectomized, and nephrectomized-parathyroidectomized rats*: Successive peritoneal rinses were carried out in each rat. Two rinses were done the first day, followed by 3 on the next day. The data are summarized in Table II. Despite the fact that a constant rinse concentration of citric acid was maintained for each rat, the wash concentration of this ion gradually decreased. This progressive drop in wash concentration of citric acid was seen in all 3 groups. It must, therefore, be assumed that the second site of citrate metabolism is extra-renal and does not require parathyroid hormone. The calcium level also dropped in each successive wash, indicating that the calcium rise was a function of the citric acid concentration of the wash.

*Discussion.* These experiments clearly demonstrate that while citric acid is able to mobilize calcium even in the absence of the parathyroid gland, this function is much curtailed after parathyroidectomy. This is borne out, first, by the drop in calcium and citric acid following parathyroidectomy and, second, by the smaller increases in calcium

TABLE II. Calcium and Citric Acid Levels in Washes of Successive Peritoneal Rinsings.

| Animals | Citric acid in rinse | Citric acid in wash | | | | Calcium in wash | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2nd | 3rd | 4th | 5th | 2nd | 3rd | 4th | 5th |
| Normal | 250 | 70 ± 7 (6) | 57 ± 6 (6) | 48 ± 6 (6) | 46 ± 5 (6) | 11.4 ± .9 (6) | 10.9 ± .7 (6) | 10.5 ± .6 (6) | 10.5 ± .4 (6) |
| | 500 | 90 ± 13 (7) | 73 ± 9 (7) | 61 ± 7 (5) | 56 ± 6 (5) | 12.5 ± 1.1 (7) | 10.9 ± .83 (7) | 10.6 ± .7 (3) | 10.8 ± .9 (5) |
| Neph | 25 | 8.8 ± 2.1 (11) | 6.2 ± 1.6 (11) | 5.7 ± 1.1 (11) | 5.8 ± 1.4 (11) | 6.8 ± .42 (11) | 6.91 ± .41 (11) | 6.82 ± .37 (11) | 6.64 ± .44 (11) |
| | 100 | 35.9 ± 5.7 (9) | 31.4 ± 4.9 (9) | 20.2 ± 5.3 (9) | 19 ± 6.1 (9) | 7.75 ± .69 (9) | 8.1 ± .73 (9) | 7.6 ± .64 (9) | 7.4 ± .67 (9) |
| | 250 | 74 ± 9 (6) | 61 ± 8 (6) | 53 ± 7 (6) | 51 ± 5 (6) | 13.8 ± 1.4 (6) | 13.1 ± 1.1 (6) | 12.4 ± .9 (6) | 12.2 ± 1 (6) |
| | 500 | 158 ± 24 (4) | 142 ± 27 (4) | 134 (2) | 123 (2) | 14.6 ± 18 (4) | 12.5 ± 1.3 (4) | 11.6 (2) | 11.1 (2) |
| Neph PTX | 25 | 16.2 ± 2.9 (11) | 13.7 ± 2.4 (11) | 10.2 ± 1.8 (11) | 7.3 ± 1.7 (11) | 3.44 ± .34 (11) | 3.19 ± .29 (11) | 3.4 ± .3 (11) | 3.23 ± .33 (11) |
| | 100 | 56.9 ± 6.3 (12) | 40.8 ± 4.2 (12) | 33.6 ± 5.5 (12) | 23.5 ± 4.7 (12) | 5.3 ± .38 (12) | 5.05 ± .35 (12) | 4.8 ± .47 (12) | 4.4 ± .38 (12) |

For notes—See Table I.

in the equilibrated washes of the parathyroidectomized rats administered citric acid. The lower calciums occurred despite the fact that these washes contained higher citrate levels than their nephrectomized controls.

Of equal interest is the disappearance of exogenous citric acid from the peritoneal wash. Experimental evidence by Martensson(11) and Freeman and Chang(12) has indicated the kidney as the main site of citrate metabolism. The evidence presented here substantiates this renal function. However, it has also been postulated that the liver, in addition to the kidney, may be able to metabolize citrate(13). These data demonstrate that in the presence or absence of the kidneys another site of citrate metabolism was activated or increased its function when citrate was administered via a peritoneal lavage. While investigation of the exact site was not undertaken, the non-effect of the parathyroids on this mechanism would tend to remove the bone from consideration. However, a definite effect of the parathyroids on citrate metabolism is seen in the slower rate of exogenous citrate loss from even the first rinse in the parathyroidectomized animal. Since this slower rate is accompanied by a decreased ability to mobilize calcium, a direct relationship of the parathyroid hormone with calcium and citric acid in bone is indicated.

The authors believe that these findings add strength to the idea that citric acid may use complexing as a means of increasing extracellular calcium. A recent article by Dixon and Perkins(14) on citric acid and bone reviewed and discussed the evidence for a calcium-citrate complex in blood, bone, and extracellular fluid. They expressed belief that such a complex would leave calcium in such a state that it would be unavailable to exert its influence on the transmission of nerve impulses. The evidence in this and other experiments supports such an idea. In the present experiment, it was noted the nephrectomized and parathyroidectomized-nephrectomized rats tolerated progressively smaller amounts of citrate and death was accompanied by violent tetany within 15 minutes of citrate administration. Data on parathyroidectomized rats in tetany from the effects of endogenous citrate acid showed plasma calcium values of 18-20 mg%(6).

While much remains to be done in determining where citric acid fits into the picture of bone metabolism, evidence is accumulating which suggests the possibility that citric acid is involved in the pathway by which bone salts are deposited and resorbed. The evidence also indicates that these processes are influenced or controlled by the parathyroid glands.

*Summary.* 1. Introduction of exogenous citric acid in a neutralized rinsing fluid into the peritoneal cavities of normal, nephrectomized and parathyroidectomized-nephrectomized male rats was studied. Normal rats survived relatively large amounts of citric acid. Nephrectomized rats were able to survive amounts of citric acid up to 0.50%; parathyroidectomized - nephrectomized rats only up to 0.20%. 2. Normal, nephrectomized, and parathyroidectomized animals showed increases in wash calcium when optimal amounts of citric acid were present in the rinsing fluid. Parathyroidectomized-nephrectomized rats given the same amount of citric acid in the rinsing fluid were found to have higher wash citric acids but lower wash calciums than their nephrectomized controls. In each group of rats the highest calcium and citric acid levels usually occurred in the first wash. Subsequent washes generally showed progressively smaller amounts of calcium and citric acid, even though the amount of citric acid in the rinsing fluid was never changed within a group throughout all the rinsings. Possible explanations are discussed.

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Received November 14, 1956 P.S.E.B.M., 1957, v94.

## Studies on Thymus of the Mammal. X. Regeneration of Irradiated Mouse Thymus.*† (23024)

CHRISTIANNA SMITH AND DOROTHY ANNE KIEFFER
*Department of Zoology, Mount Holyoke College, South Hadley, Mass.*

The source of cells which appear in the cortex of a thymus regenerating after accidental involution has been questioned because the number of observed mitoses has been considered insufficient to account for the reconstitution. Murray(1) states that repopulation of the cortex after total-body irradiation proceeds outward from the medulla. Both Murray and Baillif(2), in a study of thymuses involuted after chlorazol black E injections, consider the possibility that new lymphocytes may be derivatives of the epithelial reticulum. However, in comments on epithelial origin of the thymic lymphocytes, Downey(3) suggests that "It is possible that these conclusions can be explained by a failure to distinguish between the epithelial and mesenchymatous portions of the thymic stroma." In the present investigation, the appearance of dividing cells in thymuses regenerating after total-body sublethal dose of gamma rays (400 r) was studied in animals receiving an injection of colchicine previous to their sacrifice. Cells in mitosis appeared all through the cortex and the evidence indicates that they are mesenchymal reticular cells and medium-sized lymphocytes.

*Materials and methods.* Most of the animals used in these experiments were CAF 1 male mice, 4-5 weeks old from the Roscoe B. Jackson Laboratory. Four were of the BALB/c strain. The series studied included normal animals and mice which had been irradiated 24, 36, 52 hours, 3, 5, and 7 days previously. The dose was 400 r, Cobalt 60; aluminum filter; 38 cm from source. Time of exposure varied from 9′ 3″ at the beginning of experiments to 10′ 3″ at the end. In general, thymuses used for counting cells were fixed in Bouin's fluid and stained in hematoxylin and eosin. May-Grünwald and Giemsa solutions were also used to stain both Bouin and Helly fixed material for the study of cell types. Sections were cut horizontally at 5 $\mu$. All mitotic counts were made from animals injected subcutaneously with 1/20 cc of freshly prepared 0.5% solution of colchicine 6 hours before they were killed by cervical dislocation. As the purpose of these studies was to determine distribution of dividing cells in the regenerating thymus, time of injection had to be determined by the hour of irradiation (9 a. m.). The 24 hour irradiated mice were injected at 3 a. m., the 36 hours, at 3 p. m. and the 52 hours, at 7 a. m. All others were injected at 9 a. m. except three 3-day mice, at 7 a. m. Counts of resting and dividing cells were made with a net micrometer (56,000 cu. $\mu$, 6x7 rows) under oil immersion at a magnification of 970 times.

* This investigation was supported by a research grant from Nat. Cancer Inst., N.I.H., U.S.P.H.S.

† The authors are indebted to Dr. Philip Ives, Amherst College, for irradiation of mice.

TABLE I. Effect of a Single Total-Body Dose of 400 r Gamma Rays Followed by Injection of Colchicine on Numbers of Thymic Cells in Unit Areas.

| Mice | No. | Mean No. of cells ± S.E. Cortex | Mean No. of cells ± S.E. Medulla | Difference between means: Cortex No. ± S.E. | Cortex t | Cortex $P_t$ | Medulla No. ± S.E. | Medulla t | Medulla $P_t$ |
|---|---|---|---|---|---|---|---|---|---|
| Non-irrad. | 5 | 4155.2 ± 88.16 | 2852 ± 149.28 | | | | | | |
| | | | | 2788.8 ± 169.04 | 16.50 | <.001 | 324.4 ± 168.38 | 1.93 | >.05 <.10 |
| 24 hr | 5 | 1366.4 ± 144.24 | 2527.6 ± 77.91 | | | | | | |
| | | | | 34.2 ± 188.75 | .18 | >.80 <.90 | 306.6 ± 113.5 | 2.70 | >.025 <.05 |
| 36 " | 5 | 1400.6 ± 121.81 | 2834 ± 82.54 | | | | | | |
| | | | | 304.6 ± 153.85 | 1.98 | >.05 <.10 | 52.8 ± 170.95 | .31 | >.70 <.80 |
| 52 " | 5 | 1705.2 ± 93.95 | 2886 ± 149.71 | | | | | | |
| | | | | 43.3 ± 108.75 | .40 | >.60 <.70 | 384.5 ± 219.93 | 1.75 | >.10 <.20 |
| 3 days | 6 | 1748.5 ± 54.67 | 2501.5 ± 161.12 | | | | | | |
| | | | | 1192.7 ± 114.16 | 10.45 | <.001 | 452.5 ± 189.08 | 2.39 | >.025 <.05 |
| 5 " | 5 | 2941.2 ± 100.23 | 2049 ± 98.95 | | | | | | |
| | | | | 855.6 ± 164.62 | 5.19 | <.001 | 438 ± 176.77 | 2.48 | >.025 <.05 |
| 7 " | 4 | 3796.8 ± 130.61 | 2487 ± 146.48 | | | | | | |

TABLE II. Effect of Single Total-Body Dose of 400 r Gamma Rays Followed by Injection of Colchicine on Numbers and Percentage of Dividing Cells in Unit Areas in Thymus.

| Mice | No. | Mean No. of mitoses ± S.E. and % Cortex Mean ± S.E. | Cortex % | Medulla Mean ± S.E. | Medulla % | Difference between means: Cortex No. ± S.E. | Cortex t | Cortex $P_t$ | Medulla No. ± S.E. | Medulla t | Medulla $P_t$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Non-irrad. | 5 | 303 ± 18.58 | 7.3 | 14.2 ± 1.45 | .5 | | | | | | |
| | | | | | | 295.8 ± 18.65 | 15.86 | <.001 | 6.4 ± 3.59 | 1.78 | >.10 <.20 |
| 24 hr | 5 | 7.2 ± 1.65 | .5 | 20.6 ± 3.28 | .8 | | | | | | |
| | | | | | | 8.6 ± 3.07 | 2.80 | >.02 <.025 | 5 ± 4.04 | 1.24 | .25 |
| 36 " | 5 | 15.8 ± 2.59 | 1.1 | 25.6 ± 2.36 | .9 | | | | | | |
| | | | | | | 19.6 ± 8.21 | 2.39 | >.025 <.05 | 13 ± 9.11 | 1.43 | >.10 <.20 |
| 52 " | 5 | 35.4 ± 7.79 | 2.1 | 38.6 ± 8.80 | 1.4 | | | | | | |
| | | | | | | 130.6 ± 32.12 | 4.07 | >.001 <.005 | 26.4 ± 17.33 | 1.52 | >.10 <.20 |
| 3 days | 6 | 166 ± 31.16 | 9.5 | 65 ± 14.93 | 2.6 | | | | | | |
| | | | | | | 433.6 ± 73.25 | 5.92 | <.001 | 28.6 ± 1.73 | 1.66 | >.10 <.20 |
| 5 " | 5 | 599.6 ± 66.3 | 20.4 | 36.4 ± 8.67 | 1.8 | | | | | | |
| | | | | | | 207.1 ± 80.18 | 2.58 | >.025 <.05 | 4.9 ± 13.37 | .37 | >.70 <.80 |
| 7 " | 4 | 392.5 ± 45.08 | 10.2 | 31.5 ± 10.18 | 1.3 | | | | | | |

TABLE III. Effect of a Single Total-Body Dose of 400 r, Gamma Rays, on Percentage of Mitoses in 7 Successive Zones in Thymic Cortex from Capsule to Medulla.

| Mice | No. | % of mitoses from capsule to medulla | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Non-irrad. | 5 | 13.3 | 9.8 | 8.6 | 7.3 | 5.8 | 4.2 | 2.4 |
| 24 hr | 5 | .37 | .64 | .71 | .59 | .58 | .39 | .39 |
| 36 " | 5 | 2.6 | .86 | .98 | .77 | 1.7 | .46 | .53 |
| 52 " | 5 | 2.4 | 1.9 | 2.6 | 2.6 | 2.5 | 1.9 | .8 |
| 3 days | 6 | 11.9 | 10.8 | 10.2 | 10.1 | 9.0 | 8.3 | 6.8 |
| 5 " | 5 | 19.6 | 21.1 | 20.6 | 21.9 | 20.7 | 19.9 | 18.6 |
| 7 " | 4 | 10.7 | 12.6 | 12.2 | 11.5 | 10.0 | 8.3 | 6.5 |

Ten fields were chosen in cortex and medulla which were representative of each thymus as to cellular content and density of population. Sections were taken either 50 or 150 $\mu$ apart depending on the size of the thymus. In the cortex of the involuted thymuses, the region counted extended from capsule to medulla. However, if the width of the cortex in normal thymuses and the ones regenerating after irradiation was greater than the area of the net micrometer, 2 fields were counted: one just beneath the capsule and the other next to the cortico-medullary boundary. The data for successive zones were averaged so that they were comparable to those obtained when single fields were counted.

*Observations.* Seven and three-tenths % of cortical cells in the normal mouse thymus were in division. The most frequent type of arrested metaphase was the "ball" with scattered appearances of the "exploded" and "star" forms(4). There was a gradual decrease in numbers of mitoses toward the medulla (Table III). Dividing cells in the cortex were often grouped in cord-like arrangements or in small foci. Mitoses in the medulla were scarce.

Twenty-four hours after irradiation the thymus is characterized by a depleted and narrow cortex in contrast to a more densely populated and relatively more extensive medulla, the so-called inverted thymus. The number of cortical cells was significantly reduced (Table I). The density of medullary cells, on the contrary, was not essentially altered. In the most severely affected thymus, about 1/5 of the cortical cells remained and only 2 mitoses were seen in 10 fields examined. It was estimated that 1/3 of the undamaged cells were either mesenchymal reticular cells or medium-sized lymphocytes. In the medulla the percentage of dividing cells was 0.8%. All through these experiments the number of medullary mitoses was low though somewhat higher than in the normal organ.

The character and number of resting cells in the thymus 36 hours after irradiation had changed only slightly; there were more vacuolated cells and less debris in the cortex. As can be seen (Table III) the distribution of mitoses in this group was more irregular than in any other.

The thymuses 52 hours and 3 days after irradiation were still of the inverted type. The marked increase in percentage of dividing cells at 3 and 5 days post-irradiation was followed by an abrupt rise in total number of cells (Fig. 1, Tables I and II). By the 7th day, the number of cortical cells approached the normal and the percentage of mitotic fig-


FIG. 1. Effect of single total-body dose of 400 r, gamma rays followed by injection of colchicine on number and percentage of dividing cells in a unit area of thymic cortex.

ures had decreased. Increases in the number of cells between the 3rd, 5th and 7th days were statistically significant (Table I).

In the irradiated thymuses, most of the dividing cells were in the subcapsular or middle cortical zones (Table III). They were diagnosed as either mesenchymal reticular cells or medium sized lymphocytes; only occasionally was an epithelial cell in the cortex thought to be in mitosis and none was seen in division in the medulla.

The choice of fields in the medulla can profoundly affect the counts so that a cautious interpretation of statistical analyses has to be made. It is evident from Tables I and II that there was no clear effect of irradiation on fluctuations of medullary cells. In the 5 and 7 day series when the transition was being made from the inverted thymic pattern to the normal one of a more densely packed cortex, the indistinctness of the cortico-medullary border made the determinations of the limits of the medulla difficult. To demonstrate the importance of the choice of the field in the medulla 7 days after irradiation, counts were made in 2 thymuses in 2 regions of different densities. The following averages were secured: 2253 cells, 30 in mitosis, 1.3%; 3133 cells, 290 dividing, 9.6%. The data included in Tables I and II for the 7 day series were from the less dense, more characteristic medullary regions.

*Discussion.* In previous studies of the cellular effects of the administration of colchicine and of irradiation, the two agents were either given together(5) or the drug was injected prior to raying(6). The purpose of the present experiments in which colchicine followed irradiation was to discover where and when mitoses appeared in the regenerating thymus.

In both normal and irradiated thymuses mitoses were seen all through the cortex but fewer near the medulla. After irradiation the significant depression in numbers of cortical cells was accompanied by a similar sharp drop in the percentage of dividing cells. Even at 24 and 36 hours after irradiation by a sublethal dose, when the evidences of destructive influences were still predominant (loss of lymphocytes, presence of pycnotic and fragmenting nuclei and active macrophages, accumulation of lipids(7), strong positive reaction for acid phosphatase(8)), some cells continued to divide. At 5 and 7 days after exposure the number of cortical cells rapidly approached the normal and the peak of the curve for the mitotic cells came at the 5th day. At these times, too, the thymus was recovering its normal appearance by a diminution in the amount of lipids and by less evidence of a reaction for acid phosphatase.

The sensitivity of the cortical cells of the thymus to irradiation by a sublethal dose and the rapid recovery are well shown in Fig. 1. The difference between the average number of cells in the non-irradiated thymuses and that of the 24 hour post-irradiated thymuses and the differences between the means for the 3, 5 and 7 day ones are all statistically significant while the differences occurring during the destructive phase from 24 hours to 3 days are not (Table I). In contrast none of the variations in the counts of medullary cells within the conditions of these experiments were judged significant. The picture of the inverted thymus appears due more to the contrast of a thinly populated cortex with a relatively more densely packed medulla than to any real increase in the number of medullary cells. This comparatively stable quality of the medulla in regard to numbers of cells and percentages of mitoses correlates with its less marked reactions after irradiation as indicated by the presence of fewer lipids(7) and of less acid phosphatase than the cortex (8) during the degenerative stages.

It is believed that the cells repopulating the cortex have not migrated from the medulla to any extent as described by Murray (1) but have arisen *in situ* for the regeneration of this part of the thymus is associated with a marked increase in numbers of dividing mesenchymal reticular cells and medium-sized lymphocytes. Occasional mitoses were judged epithelial in nature but there was no reason to think that any lymphocytes were derived in this way.

In evaluating the data obtained from enumeration of colchicine mitoses, certain

factors which might influence the results must be considered: variations in state of the thymus, choice of fields, diurnal mitotic rhythm, strain of mice, time of action and dosage of the drug.

The condition of the thymus at times of irradiation and of colchicine injection is unknown. Some differences among individual as well as between different regions in the same thymus would be expected.

Diurnal variations in mitotic rhythm have been described in the epidermal(9) and respiratory(10) epithelium of mice. In the present study, none of the variations could be attributed to the time of day injections were made. Strain differences in mitotic rates have also been reported(10) but these were not found in counts from CAF 1 and BALB/c mice in the same series.

The interval chosen for action of colchicine was 6 hours, the one suggested in general for mammals(11,p.379), and the dose (1/20 cc of a 0.5% solution per animal) was within the range used by other investigators(11).

It has not been possible to correlate number of mitoses in the thymuses from normal noninjected mice with these results from animals receiving colchicine. The methods which have been used have been too dissimilar to make comparisons valid.

*Summary.* 1. The numbers of resting and dividing cells in the cortex of the mouse thymus were significantly reduced 24 hours after a single total-body dose of 400 r gamma rays followed by an injection of colchicine. Increase in the number of mitoses between 52 hours and 3 days postirradiation preceded the rise in the number of cells. The greatest number of mitoses (20.4%) appeared on the 5th day after exposure. 2. The fluctuations in the number of resting and dividing cells after irradiation in the medulla followed no regular pattern. 3. The evidence presented supports the view that the cells repopulating the thymic cortex after irradiation arise *in situ*.

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Received November 26, 1956. P.S.E.B.M., 1957, v94.

# Non-Suitability of Levator Ani Method as an Index of Anabolic Effect of Steroids. (23025)

M. E. Nimni and E. Geiger*

*Departments of Pharmacology and Biochemistry, University of S. California Medical School, Los Angeles.*

In continuation of our studies on the mechanism of testosterone induced nitrogen retention(1,2), we proposed to investigate further the effect of some recently introduced steroids with allegedly increased anabolic and decreased androgenic properties. For the screening of such compounds Eisenberg and Gordan(3) suggested a bioassay method in which weight changes of the m. levator ani (m.l.a.) of male rats are determined after injection of androgens. According to these authors the observed weight changes indicate "myotropic," *i.e.* anabolic effect of the compounds. From our observations on size and growth of this muscle under various conditions it appeared that weight changes of the m.l.a. might be a sex-linked function, the growth effect of the steroids representing an androgenic rather than a general myotropic effect. It was therefore questionable whether the m.l.a. method could be used as an indicator of the anabolic effect of steroid compounds. The present study deals with the validity of this method.

*Methods.* Male Wistar rats were kept in individual cages. The animals received Purina Chow *ad lib.* except when used in protein depletion and carbohydrate and fat repletion experiments. The composition of the *protein depletion* diet was as follows: Corn Starch 75.5%, Sucrose 10%, Butter Oil 8%, Corn Oil 3%, U.S.P. Salt Mixture #2 3%, Cod Liver Oil 0.5%. The high *carbohydrate repletion* diet contained: Casein 15%, Sucrose 8%, Corn Starch 72%, Cod Liver Oil 0.5%, Corn Oil 2%, U.S.P. Salt Mixture #2 2.5%. The *high fat repletion* diet contained: Casein 23%, Sucrose 12.5%, Butter Oil 50%, Cod Liver Oil 0.7%, Corn Oil 1%, U.S.P. Salt Mixture #2 3.9%, Celluflour 8.9%. The usual vitamin supplements were added to all 3 diets to bring them to the level indicated earlier(1). Bilateral castration was performed under light ether anesthesia. At termination of experiments the rats were killed by a blow on the head. The m.l.a. was dissected according to the technic of Hershberger, Shipley and Meyer(4), and the weight of this muscle, of the seminal vesicles, and of other organs was determined immediately with an accuracy of .1 mg. For water content determination the muscle was dried to constant weight at 80°C in an oven for 24 hours. The N-content of the dry m.l.a. was determined according to Folin-Nessler(5) after digestion in 10% $H_2SO_4$.

*Results.* In the first group of experiments we investigated the effect of a protein-free diet. The results, condensed in Table I (Group I), show that animals on protein-free diets lost body weight. The m.l.a., seminal vesicles, and kidneys participated in this general loss in body weight and were smaller than in animals on a full diet.

In further experiments (Group II), the effects of testosterone propionate (Lilly) (T.P.) on protein depleted animals were investigated. Every other day, the animals received 2.5 mg T.P. in sesame oil injected subcutaneously. Rats maintained on stock diets and protein depleted rats which received only sesame oil injections served as controls. The results indicated, in confirmation of earlier experiments(1), that the loss of body weight on protein-free diets was not affected by T.P. injections. In contrast, weight of m.l.a., seminal vesicles, and kidneys increased considerably.

The same effect could also be demonstrated in protein depleted rats which had been castrated a few days before the start of depletion. The determination of water and of nitrogen content of the m.l.a. showed further (Table I, Group II) that an increase of organ

* Supported by grant from Dairy Industry Advisory Board of State of California.

TABLE I. Effect of Testosterone Propionate on Organ and Muscle Weight of Normal and Castrated Male Rats on Protein-Free Diets.

| Group | No. of rats | Condition | Depletion period (days) | Diet | Treatment | Body wt (g) Initial | Body wt (g) Final | Wt (mg) of: Lev. ani | Water cont. lev. ani (%) | Sem. ves. | Kidney | Liver | Testic. | Diaphragm | N. cont. of dry lev. ani in mg % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | 4 | Normal | 12 | Stock | | 119 (± 13) | 182 (± 29) | 70.4 (± 30.0) | 78.5 (± .91) | 143.1 (± 59) | 1756 (± 202) | 9505 (± 1310) | 2430 (± 212) | | |
| | 4 | | 12 | Prot. free | | 118 (± 5) | 83 (± 2.7) | 13.2 (± 2.9) | 74.5 (± 1.4) | 28.7 (± 6.5) | 833 (± 53) | 4050 (± 448) | 1685 (± 272) | | |
| II | 5 | " | 15 | Prot. free | 15 mg T.P.* | 170 (± 6) | 137 (± 11) | 94.5 (± 22.9) | 78.5 (± .7) | 1103.0 (± 356) | 1307 (± 94) | 5340 (± 605) | 2590 (± 129) | 354.2 (± 46.2) | 15.5 (± 1.2) |
| | 5 | | 15 | " | | 170 (± 6) | 131 (± 6) | 33.1 (± 10.9) | 76.5 (± 1.2) | 102.2 (± 22.8) | 1137.5 (± 65.3) | 5590 (± 515) | 2355 (± 179) | 387.0 (± 52.3) | 14.8 (± .9) |
| III | 5 | Castrated | 12 | Prot. free | 15 mg T.P.* | 99 (± 7) | 71 (± 6.1) | 51.9 (± 6.7) | 78.6 (± .42) | 501.8 (± 43.5) | 948.5 (± 76.5) | 2781 (± 420) | | | |
| | 5 | | 12 | " | | 98 (± 9) | 72 (± 5.8) | 7.6 (± 2.5) | 77.8 (± 2.1) | 17.8 (± 5.2) | 783.7 (± 44.5) | 2891 (± 274) | | | |

* 2.5 mg every second day. Values in parentheses are stand. dev. from the mean.

weight after testosterone treatment was not due to water accumulation but represented a real increase of organ protein.

To ascertain how T.P. affects the growth of another muscle, we also determined (Group II) the weight of diaphragm-muscle. This muscle was selected because it could be easily dissected. The results indicated that, in contrast to the increase in m.l.a. weight, the weight of the diaphragm muscle was not affected by T.P. injections.

We then sought to determine why the weight loss of the m.l.a. on protein-free diets was relatively much larger than the loss of total body weight (Table II). One possible explanation of this phenomenon is that in rats on protein deficient diets the secretion of gonadotropic hormones decreases(6), and as a consequence the stimulus for androgen production is diminished. To investigate this possibility, normal and castrated rats were protein-depleted and during 11 days on a protein-free diet they were injected subcutaneously with 2 to 4 rat units daily of pituitary gonadotropin (Squibb). It was found (see Groups II and III, Table II) that the weight of m.l.a., of the penis, and of seminal vesicles of normal gonadotropin-treated increased considerably over weights of these tissues in control animals (Group IIIA) despite the protein depletion. In protein depleted castrated rats (Group IV), injection of gonadotropic hormone proved to be ineffective, thus suggesting that this hormone elicited its effect through the testes. To eliminate the possibility that traces of growth hormone present in gonadotropin might be responsible for the described effect, another group of animals (Group V) was treated with 10 rat units daily of Antuitrin Growth (Parke Davis). Neither total body weight nor the organ weights investigated were influenced by injection of somatotropic hormone on a protein-free diet. It should be mentioned here that an increase of the m.l.a. by injection of growth hormone had been found earlier in animals on *normal diets*(3).

Table III contains the results of experiments in which the effect of Norethandrolone on protein-depleted castrated rats was inves-

TABLE II. Effect of Growth Hormone and Pituitary Gonadotropin on Normal and Castrated Male Rats on a Protein-Free Diet for Eleven Days.

| Group | No. of rats | Condition | Treatment | Body wt (g) Initial | Body wt (g) Final | Wt (mg) of: Lev. ani | Sem. ves. | Penis | Kidney | Liver | Diaphragm |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | 5 | Castrated ♂ controls | | 137 ± 13 | 107 ± 13 | 16.5 ± 5.7 | 37 ± 12 | 94 ± 9 | 1002 ± 193 | 4580 ± 1130 | 350 |
| II | 4 | Normal | 20 rat units gonad.* | 133 ± 6 | 112 ± 1 | 52.5 ± 8.1 | 680 ± 88 | 166 ± 14 | 1168 ± 106 | 4195 ± 173 | |
| III | 4 | " | 40 " " | 134 ± 3 | 101 ± 4 | 57.5 ± 8.4 | 750 ± 229 | 179 ± 10 | 1079 ± 59 | 4006 ± 630 | 322 ± 17 |
| III-a | 5 | " | | 140 ± 2 | 115 ± 4 | 21.6 ± 1.5 | 65 ± 15 | 110 ± 8 | 995 ± 65 | 5145 ± 450 | 348 ± 25 |
| IV | 4 | Castrated ♂ | 20 " " | 131 ± 3 | 107 ± 2 | 15.7 ± 1.6 | 30 ± 5 | 95 ± 5 | 1030 ± 220 | 4000 ± 550 | |
| V | 4 | *Idem* | 100 rat units growth h. | 140 ± 4 | 108 ± 5 | 14.1 ± 1.5 | 54 ± 12.7 | 99 ± 12 | 1168 ± 83 | 5505 ± 387 | 372 ± 15 |

* Gonadotropin.

tigated in comparison with T.P. Norethandrolone has been recently introduced under the trade name of Nilevar (Searle) and, according to the manufacturer, has a high anabolic, but low androgenic, activity. For these experiments the rats were castrated at the age of 31 days and kept on stock diet for an additional 25 days. They were then protein depleted for 24 days, at the end of which time they were sacrificed. Beginning on the eleventh day of protein depletion diet, the hormones were injected daily at 2 different dose levels. The results show that Norethandrolone increased the organ weight of protein depleted castrated rats in the same way as did T.P. Body weight was not affected by the injection of these hormones.

The concluding experiments were designed to determine whether the weight loss of the m.l.a. and the seminal vesicles, which was observed during protein depletion, was reversible, *i.e.* whether these tissues could be restored to normal size after feeding the animals protein repletion diets. Rats on a depletion diet for 15 days lost on the average 22% of their weight. During the repletion period one group of rats received the high fat and the other the high carbohydrate diet in isocaloric quantities containing equal amounts of protein. The animals on high fat repletion diet were restricted to a daily intake of 7 g and the carbohydrate animals to a daily intake of 11 g, both quantities containing 44 calories and 1.6 g protein. All the offered food was consumed by the animals. Suboptimal levels of protein were fed to make more apparent any difference in the sparing effect of the other components such as carbohydrate or fat.

Table IV shows that during feeding of the protein repletion diets for 13 days the body weight increased rapidly and the weight of the investigated organs such as the m.l.a., seminal vesicles, kidney, and liver was restored to normal values. The high fat and the high carbohydrate animals responded very similarly, only the weight of the m.l.a. was significantly higher in fat-fed animals. The reasons for this difference are under further investigation.

TABLE III. Effect of Testosterone Propionate and Norethandrolone* on Organ and Muscle Weights of Castrated Protein Depleted Male Rats.

| Group† | Treatment | Weight in mg | | | | |
|---|---|---|---|---|---|---|
| | | Lev. ani | Sem. ves. | Kidney | Liver | Penis |
| I | T. P., 12 mg | 46.2 | 488 | 1100 | 4435 | 173 |
| II | Nilevar " | 37.1 | 408 | 1045 | 5365 | 150 |
| III | T. P., 2.4 mg | 44.3 | 270 | 1083 | 3873 | 153 |
| IV | Nilevar " | 33.1 | 126 | 1023 | 4313 | 138 |
| V | Controls | 4.2 | 18 | 988 | 4820 | 50 |

* Nilevar (Searle) supplied by courtesy of Searle & Co.
† Avg body wt of rats after depletion—111 (± 9) g.

*Discussion.* The preceding results show that T.P. and Norethandrolone induced growth of the m.l.a. and of some other accessory sex organs in normal and in castrated male rats, even on protein-free diets. Such deficient diets cannot support somatic growth; therefore, the animals lost weight. The fact that the growth of certain target organs can be stimulated by hormones even on such an unfavorable dietary regime must be attributed to the androgenic effect. These results also suggest that on depletion diet the proteins necessary for the hormone stimulated growth of the m.l.a. and other sex organs must be obtained from other tissues, possibly from the liver or the skeletal muscle. These findings indicate that the hormone-induced anabolism in the m.l.a. is associated with protein catabolism in other organs. Further work involving a greater number of animals would be required to identify the tissues from which the protein was shifted to the sex organs.

The fact that gonadotropic hormone and growth hormone did not affect weight of the accessory sex organs or of the m.l.a. of castrated male rats during protein depletion further indicates the specificity of the hormone effect on growth of the m.l.a.

Our conclusions that size and weight of the m.l.a. are a sex-linked function are supported by earlier observations. For example, the weight of this muscle in females is only a small fraction of that observed in adult males. After castration in males the m.l.a. loses about 60 to 70% of its original weight within 10 to 14 days. Loss of the skeletal muscle during this time is, however, usually insignificant.

Our experiments do not explain why the relative activity of the various androgenic steroids differs according to the organ selected for comparison, *i.e.* why some of them have a greater effect on the weight of the seminal vesicles, while others have a greater effect on the m.l.a. Such differences have been observed in the past, *e.g.* testosterone is ten times more active than androsterone when tested on the seminal vesicles but only 2 to 5 times as active when the comparison is based on the increase in prostate weight. On the basis of such observations and of our results, we must assume that the threshold levels, the speed of absorption and destruction and similar factors, rather than differences in

TABLE IV. Effect of a High Fat and High CHO Repletion Diets on Musele and Organ Weight of Rats Fed at an Isocaloric Level.

| Diet | Body wt (mg) | | | No. of rats | Lev. ani | % water in lev. ani | Sem. ves. | Liver | Kidney |
|---|---|---|---|---|---|---|---|---|---|
| | Initial | Depleted | Repleted | | | | | | |
| High CHO | 182 (± 11) | 140 (± 12) | 177 (± 7) | 6 | 45.6 (± 8.6) | 75.3 (± 0.8) | 281 (± 51) | 6003 (± 401) | 1348 (± 96) |
| High fat | 180 (± 13) | 144 (± 14) | 183 (± 10) | 6 | 67.7 (± 13.9) ($p = .01$) | 76.5 (± 1.3) | 219 (± 82) | 6468 (± 480) | 1373 (± 90) |
| Controls (at end of depletion period) | | | | 5 | 33.1 (± 10.9) | 76.5 (± 1.2) | 102.2 (± 22.8) | 5590 (± 515) | 1137 (± 65.3) |

androgenic and anabolic activities of the compounds, must be responsible for the differences in the effect on various organs.

*Summary.* Injection of androgens such as testosterone propionate or Norethandrolone in normal and castrated male rats promotes growth of the m. levator ani even on protein-free diets. Based on these and other experiments, it is assumed that the growth of this muscle is a sex-linked function. It is, therefore, concluded that the hormone-induced growth of this muscle is not an appropriate index for the general "myotropic," *i.e.* anabolic effects of steroid compounds.

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Received December 18, 1956. P.S.E.B.M., 1957, v94.

## Early Development of Glycogen Infiltration in Duct Epithelium of Dog Pancreas after Growth Hormone Administration. (23026)

SYDNEY S. LAZARUS AND BRUNO W. VOLK

*Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn, N. Y. City*

The original hypothesis concerning hydropic change in the pancreas advanced by Allen(1) attempted to achieve a common pathogenetic denominator for vacuolization of B cells and duct cells. B cell hydropic degeneration was considered to be due to "exhaustion of an internal secretory function" while that occurring in duct epithelium was thought to be due to "exhaustion of a proliferative function." Most subsequent workers(2,3,4) emphasized B cell hydropic degeneration as a characteristic lesion of the pancreas in experimental diabetes, resulting from "exhaustion" with the duct epithelial vacuolization as a more or less unexplained concomitant. The pathogenetic implication of the duct epithelial lesion is usually not mentioned and the exhaustion theory accepted uncritically. Toreson(5) demonstrated that hydropic degeneration in the pancreas in diabetes consists actually of the deposition of glycogen. It was suggested that the glycogen accumulation might represent merely one component of the widespread pathologic glycogen deposits found in diabetes or that it might be indicative of proliferative activity of duct epithelium with deranged islet regeneration. More recently it was shown that in cortisone treated rabbits the glycogen infiltration of duct epithelium occurs prior to and even in the absence of that in B cells(6). This observation is at variance with the exhaustion hypothesis of hydropic change unless the B cell and duct epithelium lesions are thought of as being distinctly separate and of different etiologies. However, the known facts concerning their development would negate this latter idea. It was therefore theorized that the pancreatic glycogen deposition was similar to that observed in other organs in diabetes. It was considered of importance to determine whether the time sequence observed in the cortisone-treated rabbit also occurs in other forms of experimental diabetes. The present study was designed therefore to investigate whether ductular glycogen infiltration occurs prior to or in the absence of that in B cells in the growth hormone treated dog.

*Material and methods.* The study was carried out on 27 mongrel dogs of either sex weighing between 12-15 kg. Nineteen of

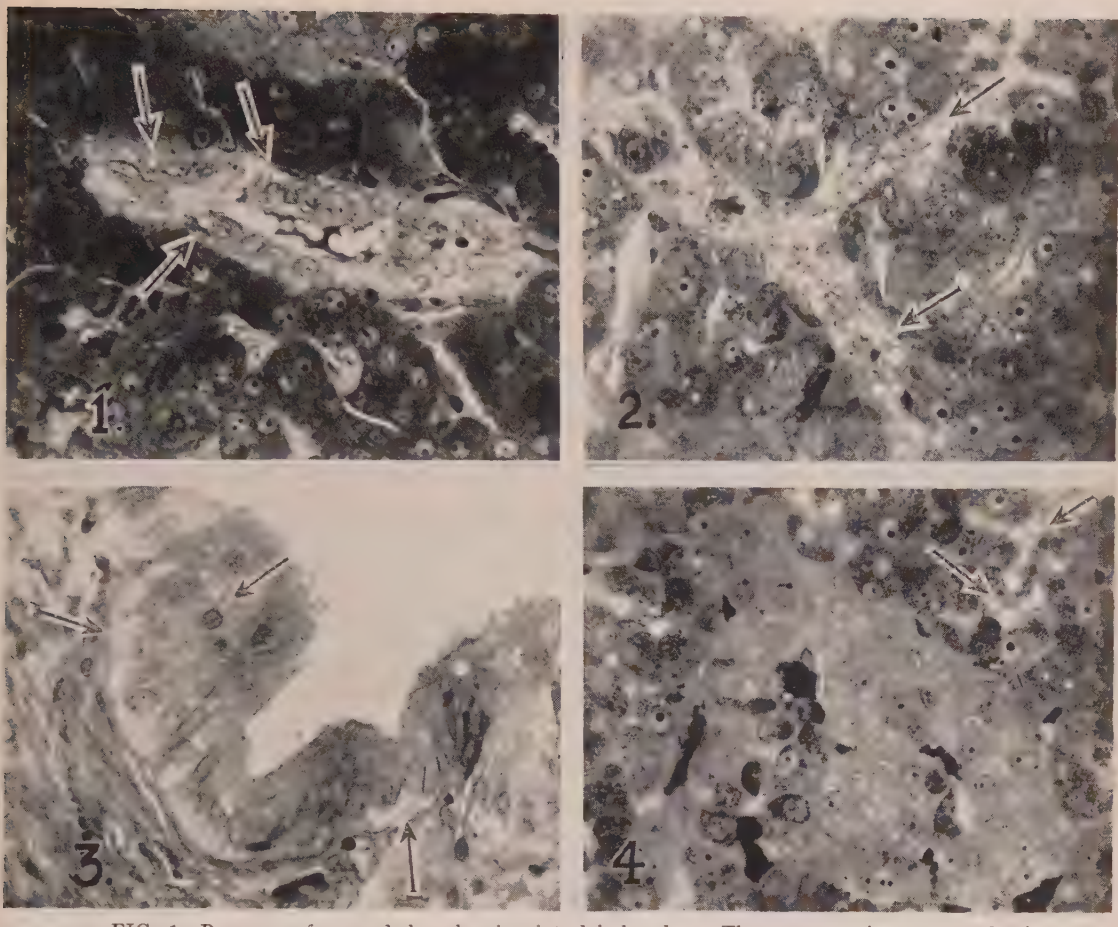


FIG. 1. Pancreas of normal dog showing intralobular duct. The arrows point to vacuoles in the cytoplasm of the epithelial cells. Masson trichrome stain. X 390.

FIG. 2. Pancreas of dog treated for 6 days with growth hormone showing increased vacuolization of intercalated duct epithelium and occasional small vacuoles in acinar epithelium. Masson trichrome stain. X 390.

FIG. 3. Pancreas of dog treated with growth hormone for 8 days showing a large duct. There is moderately increased sub- and supra-nuclear vacuolization (arrows). Masson trichrome stain. X 390.

FIG. 4. Pancreas of dog treated with growth hormone for 6 days showing intercalated duct in close contiguity (arrows) with an islet. The ductular epithelium is vacuolated. The B cells (grey in photo) are degranulated but not vacuolated. Masson trichrome stain. X 390.

these animals each received 3 mg/kg of purified anterior pituitary growth hormone* once a day by subcutaneous injection. One animal each was killed on the 1st and 2nd days, 3 on the 3rd day, and 2 each on the 5th, 6th, 7th, 9th and 11th days. Four animals died inadvertently and were not autopsied. Eight untreated dogs were used as controls. All animals received a high carbohydrate diet consisting of mashed potatoes, meat and meat gravy, and 5% glucose solution *ad libitum* instead of drinking water. The animals were killed by overdosage with intravenous nembutal and autopsies were performed immediately after death. Tissue from the tail, body and head of the pancreas was removed for histologic study and fixed in Zenker-formol solution (20%). The blocks were embedded

* The growth hormone was Armour & Co. Lot #208 provided through courtesy of Dr. Irby Bunding and Somar-A. Armour, gift of the Nat. Inst. Health.

in paraffin and were stained by the aldehyde-fuchsin(7) and the Masson trichrome methods(8). The periodic acid-Schiff method(9) controlled by diastase digestion was utilized for the demonstration of glycogen.

*Results.* A) *Normal dogs*: In sections stained by the trichrome method there were occasional small sharply defined vacuoles noticeable in the epithelium of the interlobar and intralobular ducts (Fig. 1) and also in the epithelium of the intercalated ducts. In the larger ducts with columnar epithelium they were subnuclear in position. In the smaller ducts or in the ductules the vacuoles were centrally located adjacent to the nuclei. Using the periodic acid-Schiff technic small deposits of glycogen were noted in ductular epithelium which corresponded with the cytoplasmic vacuolar changes. There was no glycogen present in the B cells of the islets.

B) *Growth hormone treated dogs*: In general the degree of vacuolization of the intralobular and of the intercalated ducts increased with duration of treatment. This increase first became apparent in an animal treated for 2 days and was usually present in animals at the 5th day and thereafter (Fig. 2). The columnar epithelium of the large interlobar ducts showed modest increase in subnuclear vacuolization (Fig. 3). B cell vacuolization was present in only 3 animals and was minimal as compared with the ductular vacuolization. The priority and preponderance of duct vacuolization was evident in many instances by the contiguity of vacuolated ducts with non-vacuolated islet tissue (Fig. 4). In several instances small vacuoles also appeared in acinar cells. P.A.S.-positive material which was removed by diastase was present in these vacuoles. Mild B cell degranulation was apparent in an occasional animal treated for 2 days. Thereafter several dogs showed very marked degranulation whereas others, even after prolonged treatment, showed little or no degranulation. Mitotic figures were sometimes found in the epithelium of ducts of all sizes and in centroacinar cells. B cell mitoses were quite rare.

*Discussion.* The present studies demonstrate that vacuolization due to glycogen deposits is sometimes present in the ductular epithelium in the normal adult canine pancreas. This finding is in accord with the previous observation that glycogen is present in the ductular epithelium of the human fetus (10) and indicates that its appearance in the diabetic pancreas is a quantitative rather than a qualitative change. It is interesting in this connection that many animal species such as the rabbit do not normally show glycogen in pancreatic ductular epithelium(6).

Our studies demonstrate that the growth hormone treated dog pancreas behaves similarly to the cortisone treated rabbit pancreas, in that there is increased ductular vacuolization due to glycogen infiltration prior to and generally in the absence of B cell vacuolization. These facts make it unlikely that a peculiarity of B cell metabolism is responsible for this glycogen infiltration. These observations also make questionable the concept that this histologic lesion is an expression of a degenerative process.

This process more logically seems to constitute merely one facet of the ubiquitously increased cellular glycogen occurring in diabetes. It is interesting in this connection that glycogen infiltration of renal tubular epithelium was considered a degenerative process until Ehrlich(11) demonstrated that the vacuoles contained glycogen. At the present time renal tubular glycogen deposits are considered a reversible metabolic phenomenon (12) which is an expression of a high urinary glucose concentration. Furthermore, the increased cardiac glycogen observed in both human and experimental diabetes is accepted as a mere reflection of the elevated blood sugar concentration(13). Similar mechanisms most probably account for the pancreatic glycogen deposition. A possible explanation accounting for glycogen in ductular epithelium at early stages would be that these cells absorb glucose from the lumen. This assumption seems to be borne out by the direct relationship between glucose concentration in blood and that in external pancreatic secretion(14).

*Summary.* The sequential development of hydropic degeneration (glycogen infiltration)

in the dog pancreas during administration of purified anterior pituitary growth hormone has been studied. Fifteen mature normal mongrel dogs each received 3 mg/kilo of growth hormone daily subcutaneously. Two each were sacrificed on alternate days for 14 days. Eight animals were used as controls. It was found that sometimes glycogen is normally present in ductular epithelium but not in B cells. Increased vacuolization (glycogen infiltration) of intralobular and intercalated ducts developed prior to and generally in the absence of the appearance of glycogen in the B cells of the islets. These findings are taken to support the hypothesis that hydropic degeneration of B cells is not a degenerative lesion resulting from "functional exhaustion" but is rather a morphologic expression of hyperglycemia similar to that seen in other organs.

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Received December 26, 1956. P.S.E.B.M., 1957, v94.

## Effect of Dietary Lipid on Rat Serum and Liver Cholesterol and Tissue Mast Cells.*† (23027)

BENJAMIN W. GRUNBAUM, JOHN R. GEARY, JR., FRANCISCO GRANDE, JOSEPH T. ANDERSON AND DAVID GLICK

*Histochemistry Laboratory, Department of Physiological Chemistry, and Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis.*

It has been shown repeatedly in man(1-2) that increase in proportion of calories from dietary lipid causes elevation of concentration of serum cholesterol. Recent reports indicate that this effect does not follow when lipid consists of certain vegetable oils(3). Moreover certain oils rich in linoleic acid have been reported to produce a significant decrease of serum cholesterol concentration in man(4-8). In view of differences in cholesterol metabolism between man and rat, the present study on the rat is concerned first with the effect of different fat-diets on cholesterol concentration in serum lipoproteins and in liver.

Since heparin has a "clearing" action on lipemic serum, consideration was also given to the possibility that heparin might serve to some degree as a physiological regulator of blood lipid. Tissue mast cells contain heparin, and the question is whether their number, intact or undergoing disruption, is influenced by nature and concentration of blood lipid. This possibility was examined by counting mast cells in external ear tissue of rats maintained on various diets for study of serum and liver cholesterol.

*Methods.* Adult male albino rats of Sprague Dawley strain were used. The age-

* This study was supported in part by research grant from N.I.H., U. S. Public Health Service, and by grant from Louis W. and Maud Hill Family Foundation.

† The authors wish to express their appreciation for the technical assistance given by Georgiana Psik and Nedra Whittaker, and for pathological examinations of specimens by Dr. Lee Wattenberg.

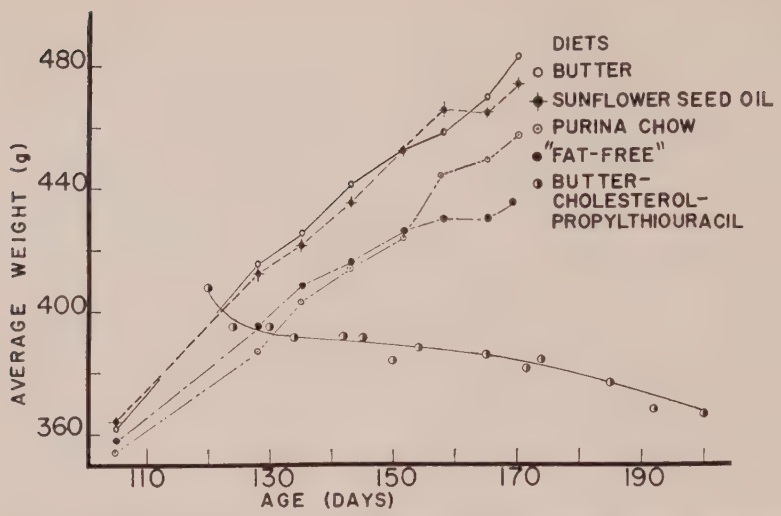


FIG. 1. Weight changes of rats maintained on different diets. Ralston Purina Chow diet: stated to have no less than 5% fat, 8 rats. "Fat-free" diet: Nutritional Biochemicals Co., 8 rats. Butter diet: 79% Purina Chow, 21% butterfat, 8 rats. Sunflower seed oil diet: 75% Purina Chow, 25% sunflower seed oil (iodine no. 131), 8 rats. Butter-cholesterol-propylthiouracil diet: 75% Purina Chow, 21% butterfat, 5% cholesterol (0.1% propylthiouracil added to mix), 16 rats.

weight relationships for animals on different diets are given in Fig. 1. Food and water *ad libitum* was furnished. Blood was obtained from tail or, for last sample, when animal was sacrificed, by heart puncture. Total serum cholesterol was estimated by the method of Abell, *et al.*(9) as modified by Anderson and Keys(10). Electrophoretic separation of lipoproteins was carried out by the method of Williams, *et al.*(11) with a Durrum Spinco, model R, apparatus (Beckman Instruments, Belmont, Calif.). From each sample of serum 20 $\mu$l were taken for protein electrophoresis and 100 $\mu$l for lipoprotein electrophoresis. Barbiturate buffer (Spinco buffer, B-2) of pH 8.6 was used. With rat serum, best separation was achieved when 11.5 ma at 150 volts were applied for 16.5 hours. After drying, the paper strip containing 20 $\mu$l was stained with bromphenol blue (Spinco dye, B-1) to indicate the various protein zones, and the strip with 100 $\mu$l was stained with Sudan black (2 g in 1 l 60% alcohol, refluxed 3-4 hours, decanted after cooling) to show lipoprotein zones. Two additional paper strips which carried 100 $\mu$l serum each, were subjected to electrophoresis and drying but not staining. The 20 $\mu$l of serum were deposited in one application on strip by means of striper supplied with the apparatus, while 100 $\mu$l in a graduated micropipette were delivered in 5 approximately equal portions onto paper strip by means of the striper. The unstained strips were cut into segments and analyzed for cholesterol using the stained strips as references. A piece 40 mm long, was cut from paper behind the starting line, where no serum components were present, to serve as a blank. Extraction of cholesterol from paper segments followed procedure of Anderson and Keys(10). The final volume of colored solution was 0.6 ml and was measured in Beckman DU spectrophotometer using Lowry-Bessey microcuvettes having 1 cm light path. Liver cholesterol was estimated in pieces (4 to 5 g) removed immediately after bleeding the animal. Liver samples were digested in 2 ml 30% KOH at about 90°C for 0.5 to 1.5 hours, or until the liver completely disappeared. After diluting to 50 ml with distilled water, 2 ml aliquots were added to 2 ml of absolute alcohol and 4 ml of petroleum ether. After shaking for a minute, the petroleum ether layer was removed. Extraction of the aqueous solution with a 4 ml portion of petroleum ether was repeated.

TABLE I. Effect of Diet on Total Cholesterol in Rat Serum and Liver.

| Diet | Mean cholesterol (mg %) ± stand. error of mean, Weeks on diet 1 | 9 | 9 |
|---|---|---|---|
| | Serum | | Liver |
| Purina chow | 65 ± 3 | 58 ± 3 | 190 ± 12 |
| "Fat-free" | 85 ± 7* | 73 ± 3† | 192 ± 4 |
| Butter | 84 ± 4† | 75 ± 4* | 215 ± 8 |
| Sunflower seed oil | 88 ± 4† | 84 ± 3† | 366 ± 40† |

* Significance of difference from corresponding Purina group, P = 0.05.
† Significance of difference from corresponding Purina group, P = 0.01.

The solvent was evaporated, the lipid was dissolved in alcohol-acetone(1:1), and cholesterol was precipitated as digitonide using aluminum chloride and ammonium hydroxide to speed the process as suggested by Brown, *et al.*(12). The method of Foldes and Wilson(13) was used to complete cholesterol determination. Because of high concentration of mast cells in the external ear and the ease of obtaining these specimens, samples of wide, mid-portion were routinely taken from each rat at time of sacrifice. Sections 12 $\mu$, were prepared and stained with toluidene blue by the method of Smith and Atkinson(14) so that auricular cartilage appeared as a dark, central band with subcutaneous tissue and skin on each side. The mast cells in random fields, 0.17 x 0.17 mm, including subcutaneous tissue both dorsal and ventral to auricular cartilage were counted (Table IV).

*Results.* Mean weight curves for experimental groups are presented in Fig. 1. Animals on "fat-free" diet showed retarded growth after 7 weeks on this diet. Rats on butter-cholesterol-propylthiouracil diet showed progressive weight loss from beginning of experiment. In spite of weight effects all animals had a normal and healthy appearance.

Table I shows changes in serum and liver cholesterol for the first 4 groups of rats.

Rats on diets other than Purina chow showed increase in serum cholesterol concentration compared with the latter group. No change of liver cholesterol was observed in animals on "fat-free" and butter diets compared with those on Purina chow, while rats receiving sunflower seed oil showed a significant increase.

Serum and liver cholesterol of animals on butter-cholesterol-propylthiouracil diet is given in Table II. Both serum and liver cholesterol values were higher than those found on diets listed in Table I, and serum cholesterol increased with time. These increases are significant when comparisons are made with data from rats on Purina chow. All animals indicated in Table II had large fatty livers.

Cholesterol in serum lipoprotein fractions was measured on serum samples from 1 or 2 animals from each experimental group. Results are presented in Table III.

Distribution of cholesterol among serum protein fractions in the rat is strikingly different from that observed in man. While man carries about 80% of total serum cholesterol in the beta globulin(10), rats, on the Purina chow diet, had only about 20% in this fraction. These findings agree with those of Fidanza and Cresta(15). Changes in dis-

TABLE II. Total Cholesterol in Serum and Liver of Rats on Butter - Cholesterol - Propylthiouracil Diet.

| No. of rats | Wk on diet | Mean cholesterol (mg %) ± stand. error of mean, Serum | Liver |
|---|---|---|---|
| 5 | 3 | 177 ± 20*† | 568 ± 159* |
| 5 | 6 | 243 ± 24* | 820 ± 162* |
| 6 | 11 | 306 ± 23* | 675 ± 166* |

* Significance of difference from Purina group (9 wk on diet), P = 0.01.
† One sample lost.

TABLE III. Distribution of Total Cholesterol among Serum Protein Fractions Separated by Paper Electrophoresis.

| Diet | Wk on diet | Cholesterol (% of total) Albumin | $\alpha$-globulin | $\beta$-globulin |
|---|---|---|---|---|
| Purina chow | 9 | 30 | 51 | 19 |
| "Fat-free" | 9 | 50 | 35 | 15 |
| Butter | 9 | 37 | 47 | 16 |
| Sunflower seed oil | 9 | 44 | 40 | 16 |
| Butter-cholesterol-propylthiouracil | 3 | 20 | 53 | 27 |
| *Idem* | 6 | 23 | 57 | 20 |
| " | 11 | 29 | 45 | 26 |
| "Normal" human serum | | 12 | 7 | 81 |

tribution observed in samples of serum from animals on "fat-free," butter, and sunflower seed oil diets appear to be unimportant. This indicates that increased cholesterol caused by special diets is distributed among all protein fractions.

It is evident (Table III) that the albumin zone contained a considerable fraction of total cholesterol. When the paper strips were stained with Sudan black, 2 distinct zones were visible corresponding to albumin and alpha globulins. The Sudan black stain is not sensitive enough to reveal quantities of lipids such as found in beta globulin zone of rat serum. In human serum beta globulin stains intensely, albumin less, and alpha globulin very little if at all.

In contrast with its action in man(6,8), sunflower seed oil at the 25% level in rat diet, produced an *elevation* of blood serum cholesterol similar to that observed in the group of animals receiving butterfat. Sunflower seed oil also caused an increase in liver cholesterol compared with Purina chow alone or plus 21% butterfat. The rats receiving "fat-free" diet showed an increase of blood serum cholesterol but no significant change of liver cholesterol. These results differ with those reported for fat-free diet by Alfin-Slater *et al.*(16), who found a decrease in blood serum cholesterol and an increase in liver cholesterol. The fat-free diet in our experiment differed from that of Alfin-Slater *et al.* in containing, respectively, 16% *vs.* 4% cellulose, 0.60% *vs.* 0.085% choline, and in continuing for 9 *vs.* 20 weeks. It appears from our experiment that elevation of serum and liver cholesterol in animals receiving sunflower seed oil can not be attributed to deficiency of essential fatty acids.

Addition of cholesterol and propylthiouracil to Purina-plus-butter diet enhanced hypercholesterolemia and caused an increase of cholesterol content of the liver. On fractionation of serum proteins by electrophoresis and subsequent determination of cholesterol in various protein fractions (Table III), it was found that with prolonged high blood cholesterol the proportion of cholesterol in the albumin zone decreased slightly while that in the beta globulin region increased slightly. Results on rats fed butter-cholesterol-propylthiouracil are similar to those of Page and Brown(17) on hypothyroid rats whose diets were supplemented with cholesterol. These authors reported increase of serum beta lipoprotein but no data were given for distribution of cholesterol among protein fractions.

TABLE IV. Mast Cell Counts on Tissue Sections from Ears of Rats on Different Diets. 100 microscopic fields counted for each diet (20 fields/rat, 5 rats/diet).

| Diet | Mean No./field | Stand. dev. | Stand. error of mean |
|---|---|---|---|
| Purina chow | 13.2 | 3.6 | .4 |
| "Fat-free" | 12.8 | 3.8 | .4 |
| Butter | 12.1 | 2.9 | .3 |
| Sunflower seed oil | 11.6 | 2.9 | .3 |
| Butter-cholesterol-propylthiouracil | 12.3 | 3.1 | .3 |

Recently Fillios *et al.*‡ reported development of atherosclerosis in rats receiving purified diets supplemented with cholesterol, sodium cholate and thiouracil, and showing marked hypercholesterolemia and hyperbeta lipoproteinemia. No gross lesions were seen in arteries of any animals employed in our study. Both rat and dog, animals considered resistant to atherogenesis(17), show high levels of alpha and lower levels of beta lipoprotein(18).

It is apparent from Table IV that the different diets had no appreciable influence on mast cell counts in rat ears, and therefore it appears that a response in mast cell number does not occur under these conditions associated with varied levels of serum and liver cholesterol. Furthermore, no difference in proportion of abnormal or disrupted mast cells was found.

*Summary.* 1) An increase of blood serum cholesterol has been observed in rats subsisting on Purina chow diet when supplemented with butterfat, or sunflower seed oil. Animals on sunflower seed oil also showed increase of liver cholesterol. Rats on "fat-free" diet showed increase in serum cholesterol but no change in liver cholesterol. When Purina chow-butter diet was supplemented

‡ Reported in *Fed. Proc.*, 1956, 550.

with cholesterol and propylthiouracil, an even greater increase in both serum and liver cholesterol was found. 2) Separation of serum protein fractions by paper electrophoresis revealed that most of the cholesterol was associated with albumin and alpha globulin fraction in the rat, whereas, in man, the greatest proportion of cholesterol is found in the beta globulin fraction. This same general distribution was observed in all dietary groups regardless of their serum cholesterol levels, although rats receiving butter-cholesterol-propylthiouracil diet showed a slight shift of cholesterol toward the beta globulin fraction. 3) No diets induced a demonstrable change in mast cell count on tissue of rat ear.

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Received January 16, 1957. P.S.E.B.M., 1957, v94.

## Experimental Production of Tuberculous Pericarditis.* (23028)

R. W. POSTLETHWAIT, J. R. WILSON, M. H. SALEM, D. C. GROSSKREUTZ, W. C. SEALY, AND D. T. SMITH

*Departments of Thoracic Surgery and Microbiology, School of Medicine, Duke University, and Veterans Administration Hospital, Durham, N. C.*

We were interested in producing tuberculous pericarditis in animals to study frequency of development of the constrictive phase, to determine the effect of antituberculous drugs and to investigate the proper time for pericardiectomy. Prior to this study, no reference could be found concerning experimental tuberculous pericarditis, but after completing our work the report of Kinoshita(1) was found. Kinoshita produced tuberculous pericarditis in rabbits with or without preceding sensitization, and his results were similar to those noted below.

* Supported in part by grant from N. Car. Medical Fn.

*Procedure.* New Zealand white rabbits weighing approximately 5 kg were sensitized by subscapular injection of 1 cc dried heat killed bovine tubercle bacilli. Three mg of the ground material were suspended in each cubic cm of Bayol F. From 6 to 12 weeks later, skin tests with commercial tuberculin were carried out and approximately one-third of the original group discarded because of negative skin tests. In the remaining sensitive animals, 0.5 cc of milky suspension of

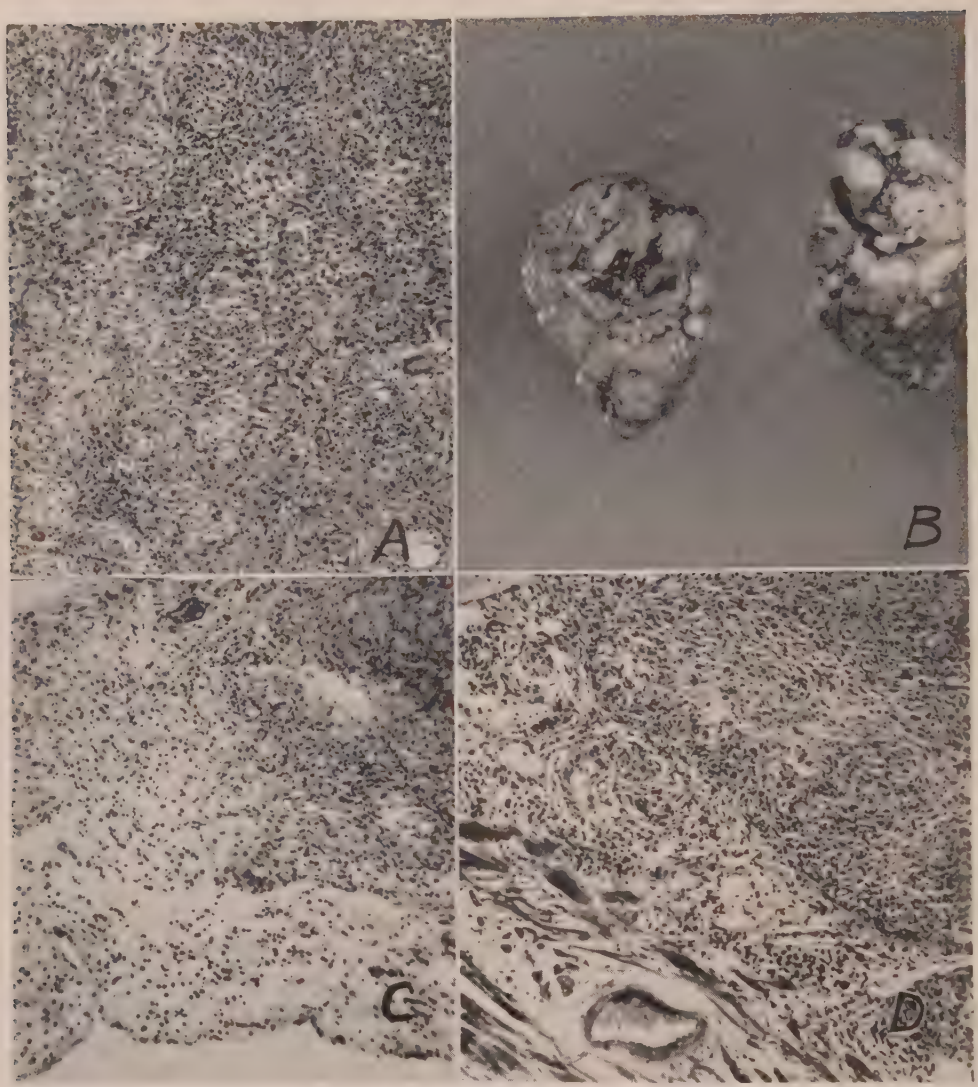


FIG. 1. (a) Tubercle formation in animal sacrificed at 4 weeks (X50). (b) Hearts of animals sacrificed at 10 weeks (left) and 12 weeks (right) showing diffuse epicardial involvement. (c) Pericardial sac from heart on right in b. Edema, cellular infiltration and increased vascularity present (X50). (d) Section from epicardium, same animal as c. Note reaction in cardiac muscle (X50).

live bovine tubercle bacilli were injected into the pericardium through small incision in the left fourth intercostal space. Twenty-five animals were injected. Two died and 2 were sacrificed on second postoperative day, one died on third day, 3 animals were sacrificed at end of 1, 2, 3, and 4 weeks and 2 at end of 6, 8, 10, and 12 weeks. Preoperative chest x-rays and electrocardiographs were made and these were repeated at time of sacrifice. No special care and no drugs were given.

*Results.* All animals showed varying degrees of inflammatory reaction grossly. In 5, this was classified as slight, 13 as moderate, 4 as severe, and 3 as very severe. The earliest reaction was a loss of normal sheen of pericardium and epicardium with accumulation of cloudy, sometimes brownish, fluid. Later roughening of these surfaces was noted, and by the end of 2 weeks, grayish white,

raised, slightly irregular patches were seen. These areas varied in number, size, and height, but were present in all animals after the first week. In the slightest reactions found, a number of these granuloma were noted along coronary vessels and over the auricles. In the more extensive disease, patches were found over the epicardium of the ventricles, and lastly over the inner layer of the pericardial sac. In the later and more severe reactions, the entire heart was a nodular mass enclosed in a thickened sac. The fluid varied in amount and color; it was usually cloudy yellow in the later groups, but not infrequently hemorrhagic. Although loose fibrinous adhesions were occasionally noted, no dense adherence of the 2 layers developed. Cultures made in 12 animals were positive in 8.

Microscopically, the pericardium showed edema and increased vascularity in those with slight reaction. The tubercles showed round conglomerations with large macrophages in the center, epithelioid cells and lymphocytes in the surrounding zone and occasional giant cell formation. Capillary proliferation was noted in some areas. No acid-fast stains were done.

The radiographic studies were not particularly satisfactory although gross enlargement could be noted at times. The electrocardiographic tracings frequently showed T wave changes, but were not diagnostic.

*Discussion.* At the time of sacrifice, anesthesia was obtained and the chest opened so that cardiac action might be observed directly. In those animals with the more severe reaction, no constrictive pericarditis had developed, but restriction of cardiac activity was evident. Based on the uniformly positive results obtained in this series, and the similar findings by Kinoshita, further studies over longer periods of observation should be carried out, to obtain more information regarding tuberculous pericarditis.

*Summary.* Tuberculous pericarditis was produced in sensitized rabbits by the intrapericardial injection of live bovine tubercle bacilli.

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Received January 28, 1957. P.S.E.B.M., 1957, v94.

## Experimental Hypertension in Rats Produced by Dietary Condiments. (23029)

LAURENCE W. ROTH AND JACKSON BLAIR
*Dept. of Pharmacology, Abbott Laboratories, N. Chicago and Babies Hospital, Cleveland, O.*

Blair(1) reported that, in a group of persons undergoing routine physical examination, he had observed some degree of correlation between occurrence of hypertension with excessive use of condiments in the daily diet. He reported 50 cases, presenting the theory that excessive use of spices and condiments, especially pepper, mustard and ginger, is a common cause of hypertension; and stated his opinion that hypertension may be produced experimentally in animals by repeated feeding of these spices and condiments, and also that the damage produced may be irreversible. In pilot experiments (in cooperation with J. E. Owens, unpublished data) it was found that rats, maintained on diets to which pepper, mustard and ginger had been added, developed hypertension. However, due to circumstances beyond his control, this work was not pursued further. Personal correspondence led to resumption of our studies on experimental hypertension.

*Methods.* Female rats (200-250 g) were used. Blood pressure was determined by photoelectric plethysmographic method of Kersten *et al.*(2). Each value recorded represents the average of at least 4 consistent readings in a single test period. Initially, the condiments were mixed in powdered breeder's diet. Later, for reasons to be discussed, a

TABLE I. Systolic Blood Pressure of Rats on Diets Containing Condiments.

| Diet | Mean systolic blood pressure* (mm Hg) ± S.E. | | | |
|---|---|---|---|---|
| | Initial | 7 mo | 10 mo | 12 mo |
| Control | 122 ± 7.5 | | 115 ± 7.1 | |
| I | | 154 ± 11.9 | 122 ± 11.4‡ | 174 ± 7.6† |
| II | | 152 ± 9.7 | 165 ± 6.3 | 156 ± 10.4 |
| III | | 171 ± 7.4 | 173 ± 15.0 | 164 ± 4.5 |

* Blood pressure averages from 5-7 animals, except (†). Value for each animal an avg of at least 4 consistent readings.

† Avg of 2 animals, only survivors at 12 mo.

‡ Only experimental value not significantly different at 1% level.

commercial producer* cooperated in supplying a standard diet in which the condiments were mixed before extrusion of the pellets. All condiments were standard table grade, obtained from a wholesale grocery supply house. Diet I contained 2% pepper, Diet II contained 1% each of mustard and ginger, and Diet III contained 2% pepper, 1% mustard and 1% ginger. Blood pressures and weight of each animal were obtained periodically. At the end of one year, representative animals were sacrificed for a complete gross and microscopic histopathological study.

*Results.* The original powdered diets produced a high incidence of respiratory involvement, presumably because of irritant qualities of the powdered condiments, which were inhaled during feeding. Before the pellets were obtained, several deaths with pulmonary symptoms occurred. This respiratory involvement was no longer observed when the pellet diet was instituted.

Table I shows average blood pressure reading obtained during the experimental period, for all diets. The average control value obtained before diets were instituted was 122 mm Hg ± 7.48. One group was maintained continuously on Diet I (pepper) for one year. It will be noted that there was a decline in blood pressure level at 10 months to essentially normal values. From then until the end of the year all rats lost weight, 3 died, and the 2 which survived were sacrificed for pathological study. Their final pressures before autopsy were 168 and 180 mm Hg.

Another group was maintained on Diet II (mustard and ginger) for one year. These animals showed a similar rise in blood pressure (Table I), but did not exhibit a secondary decline as observed with Diet I. They did not lose weight, and appeared healthy through the experimental period.

Animals on Diet III (pepper, mustard and ginger) were started earlier, and were studied in somewhat greater detail. The average blood pressure after only 8 weeks on the diet was 167 mm Hg. Withdrawal to non-condiment control diet for 3 weeks was accompanied by a decline to 157 mm Hg. Return to Diet III for 5 weeks elevated the pressure to 168 mm Hg. Thereafter, temporary withdrawal did not reverse the pressure trend, and after a cumulative total of 8 more weeks of intermittent use of Diet III the average blood pressure was 176 mm Hg. At that point the group was permanently withdrawn and kept on control diet. Throughout a period of 7 months the mean blood pressure values averaged 171 mm Hg (range 165-183) over 14 periodic readings. The last reading at 16 months was 172 mm Hg. This implies that a permanent hypertensive state had been established. The use of experimental antihypertensive drugs produced a lowering of blood pressure to the same extent as obtained in rats with experimental renal (Grollman) hypertension.

*Pathologic findings.* Kidney samples from each diet series were submitted for histological examination. Two rats were from Diet I, 3 from Diet II and 3 from Diet III.

Grossly, kidneys from rats fed the above-mentioned diets exhibited some congestion but were otherwise essentially negative. There was no evidence of cortical atrophy, surface scarring or vascular sclerotic changes. Blocks of tissue from these organs were fixed

* The Vi-D-Co., Marion, Ind.

in formalin and stained with hematoxylin and eosin for general topography, with Schiff's Periodic Acid for changes in basement membrane and capsule, and with Unna's Orcein stain for changes in elastica of blood vessels.

Microscopically, the parenchyma, stroma and the renal-vascular apparatus exhibited no changes compatible with any drug action. The tubular system was essentially negative, no abnormal changes were seen in the elastica of blood vessels and the PAS reaction was within normal limits. Final diagnosis was "essentially negative kidney findings."

*Discussion.* It would appear that mustard or ginger contains some factors capable of producing changes leading to a hypertensive state in the rat, while pepper has a factor which is less effective, though possibly additive when combined with other condiments. As a collateral observation, it was noted that the fur of the animals on Diets II and III was much softer to the touch than normal rats, while those receiving pepper failed to show this effect. The benefit probably derived from additional sulfur available in the mustard. All groups exhibited a temporary reluctance to accept the diets, but soon resumed normal intake as judged by an essentially normal growth curve. The latter part of the year for the pepper group was the only exception to this, and the cause of their decline is not known at present.

Future experiments may explore the possibility of extraction of the condiments in an attempt to isolate a single identifiable factor which will induce the hypertensive state.

In view of the essentially negative findings in the examinations of the kidneys, the authors are still at a loss to explain the mechanism of action in the production of hypertension.

*Summary.* Continued feeding of pepper, mustard and ginger in various diet combinations results in appearance of a statistically significant hypertension in rats. The greatest elevation occurred in a diet containing all 3 condiments; the hypertension from this diet persisted for months after the diet was discontinued. Other combinations were less effective, and work is continuing to determine if any single representative contains the necessary and sufficient factor for the production of hypertension.

The authors gratefully acknowledge the cooperation of R. J. Stein for his pathologic evaluation of the kidneys, and M. R. Sisler for technical assistance.

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